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STUDIES OF DRUG DISPOSITION IN HEMODIALYSIS PATIENTS - IMPACT OF GENETICS, INFLAMMATION AND VITAMIN D

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ABSTRACT

Chronic kidney disease (CKD) patients are at a high risk for drug adverse effects due to accumulation of drugs, which normally are excreted via the kidneys. It is believed that drugs that are metabolized by the liver are safe to prescribe in normal doses to end-stage renal disease (ESRD) patients. However, emerging data show that kidney failure itself can affect enzymes and drug transporters. Thus, the concentration of many drugs that normally are metabolized by the liver increases significantly in CKD patients.

A common feature of the uremic phenotype that may affect drug metabolism is persistent inflammation. Inflammation has been shown to reduce the activity of both drug metabolizing enzymes and transporters. The increased risk of drug-drug interaction implicates the necessity of safer and individualized adjusted drug dosing methods. Factors that are important I this matter need to be identified. We have studied the impact of genetic polymorphism, inflammation and vitamin D on drug metabolism in prevalent hemodialysis patients.

In paper I we studied the influence of three factors on drug disposition: genetic polymorphism, impaired renal excretion of drug metabolites and the possible elimination by hemodialysis (HD), using codeine as a test substance. Based on the genotyping of three CYP2D6 polymorphisms in 228 HD patients, 9 extensive metabolizers (EMs) and 2 poor metabolizers (PMs) were given a single oral dose of 50 mg codeine phosphate. Plasma levels of its metabolites codeine-6-glucuronide (C6G), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) were determined after 2, 4, 6, 8 and 24 hours (beginning of the HD session), respectively and again after 4 hours of HD (28 hours). Our results showed that the formation of the codeine metabolites M3G and M6G was dependent on the CYP2D6 genotype as previously shown in healthy subjects. The elimination of the glucuronides in these patients was absent until the dialysis was performed. Our data suggest that CYP genotypes need to be taken into consideration, when drugs metabolized by polymorphic CYPs are prescribed in HD patients.

In Papers II and III, we studied the effect of inflammation on cytochrome P450 3A4 (CYP3A4) activity using alprazolam and quinine as test drugs, both known substrates of CYP3A4. Twenty-six prevalent HD patients were included in study II and 44 prevalent HD patients in study III. Each patient received a single dose of the test drug and concentration of the test drugs and their metabolites were measured at the beginning of the next HD session. Inflammatory markers were followed prior to the drug test.

The ratio of unconjugated alprazolam/4-hydroxyalprazolam and 4β-OH-cholesterol/cholesterol (paper II) and ratio of quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol (paper III) were used as surrogate markers of CYP3A4 activity. In both studies, we found significant correlation between inflammation and CYP3A4 expressed as alprazolam/4-hydroxyalprazolam and quinine/3-OH-quinine respectively, but not with 4β-OH-cholesterol/cholesterol. Our results suggest that inflammation down-regulate the activity of CYP3A4. Further studies are needed to confirm this finding and to assess the extent and duration of the effect of inflammation. CYP3A4 metabolizes almost 50% of all drugs currently used in health care. Thus, if persistent inflammation affects CYP activities this will have important clinical impact by increasing the risk of drug-drug interaction and adverse side effects.

In paper IV we studied the impact of 25-OH-cholecalciferol on CYP3A4 activity. Eight prevalent hemodialysis (HD) patients completed the study. The concentration of 25-OH-vitamin D_3 was measured at the start of the study and subsequently once a month. Subjects were given a daily dose of 800-1600 IU of 25-OH-vitamin D_3 until its concentration was close to 75 nmol/L. A single dose of 100 mg quinine was given to each subject. Concentration of quinine and its metabolite 3-OH-quinine were measured 12 hours after drug intake at the beginning of the next dialysis. Ratio of quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol were used as surrogate markers for CYP3A4 activity. Concentrations of inflammatory markers were measured at the beginning and at the end of the study. Our results show no significant association between 25-OH-vitamin D and CYP3A4 activity expressed as either quinine/3-OH-quinine or 4β-OH-cholesterol/cholesterol. Our finding suggests that short term supplementation of 25-OH-vitamin D_3 does not affect CYP activity.

LIST OF PUBLICATIONS

1. Hadi Molanaei, Juan Jesus Carrero, Olof Heimbürger, Louise Nordfors, Bengt Lindholm, Peter Stenvinkel, Ingegerd Odar-Cederlöf and Leif Bertilsson. Influence of the CYP2D6 polymorphism and hemodialysis on codeine disposition in patients with end-stage renal disease. *Eur J Clin Pharmacol (2010) 66:269–273*

2. Hadi Molanaei, Peter Stenvinkel, Abdul Rashid Qureshi, Juan Jesús Carrero, Olof Heimbürger, Bengt Lindholm, Ulf Diczfalusy, Ingegerd Odar-Cederlöf and Leif Bertilsson. Metabolism of alprazolam (a marker of CYP3A4) in hemodialysis patients with persistent inflammation. *Eur J Clin Pharmacol (2012) 68:571–577*

3. Hadi Molanaei, Abdul Rashid Qureshi, Olof Heimbürger, Bengt Lindholm, Ulf Diczfalusy, Björn Anderstam, Leif Bertilsson and Peter Stenvinkel. Inflammation down-regulates cytochrome P450-catalysed drug metabolism in hemodialysis patients. (Submitted)

4. Hadi Molanaei, Abdul Rashid Qureshi, Olof Heimbürger, Bengt Lindholm, Ulf Diczfalusy, Erik Eliasson, Leif Bertilsson and Peter Stenvinkel. No differences in quinine metabolism in hemodialysis patients before and after treatment of vitamin D deficiency. (In manuscript)

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding Cassette
AGEs	Advanced glycation end products
APD	Automated peritoneal dialysis
AUC	Area under the curve
BMI	Body mass index
CAPD	Continuous ambulatory peritoneal dialysis
CAR	Constitutive androstane receptor
CHF	Congestive heart failure
CKD	Chronic kidney disease
CVC	Central venous catheter
CVD	Cardiovascular disease
CRP	C-reactive protein
CYP	Cytochrome P 450
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
EM	Extensive metabolizer
GFR	Glomerular filtration rate
HD	Hemodialysis

hsCRP	High sensitive C-reactive protein
ICAM-1	Intercellular adhesion molecule
IL-1	Interleukin-1
IL-6	Interleukin-6
IM	Intermediate metabolizer
MRP	Multidrug resistance protein
NADPH	Nicotinamide adenosine dinucleotide phosphate
OATPs	Organic anion transporting polypeptides
PCVD	Peripheral or cerebrovascular disease
PD	Peritoneal dialysis
PEW	Protein-energy wasting
P-gp	P-glycoprotein
PM	Poor metabolizer
PTX3	Pentraxin 3
PXR	Pregnane X-receptor
RRT	Renal replacement therapy
SLC	Solute carrier membrane transporter protein
SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
TNF	Tumor necrosis factor

UGT	UDP-glycosyltransferase
V_d	Volume of distribution
WBC	White blood cell

1. INTRODUCTION

1.1 **Chronic Kidney Disease (CKD)**

1.1.1 **Definition**

CKD is defined as kidney damage or decreased glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for 3 months or more, irrespective of cause (1). To define kidney function CKD is divided into five different classes/stages. Stage 1 is considered a normal kidney function and stage 6 as end stage renal disease (ESRD) **(Table 1)**.

Table 1: CKD stages with corresponding GFR levels

CKD stage	**GFR (mL/min/1.73 m^2)**	**Definition**
CKD 1	>90	Normal
CKD 2	60-90	Mildly decreased
CKD 3a	45-59	Mildly to moderately decreased
CKD 3b	30-44	Moderately to severely decreased
CKD 4	15-29	Severely decreased
CKD 5	< 15	End-stage renal disease

1.1.2. **Prevalence of CKD, symptoms and complications**

CKD is recognized as an increasing global public health problem. Different studies worldwide have shown that approximately 10 % of the population have some degree of CKD (2-4). Je et al. showed that the prevalence of low, moderately increased, high and very high CKD risk prognosis in the Korean adult population was 92%, 6.3%, 1.1% and 0.6% respectively (3). In a similar study conducted by Chen et al. the age-standardized prevalence of GFR 60 to 89, 30 to 59, and <30 mL/min/1.73 m^2 were 39.4%, 2.4% and 0.14% in Chinese adults aged 35 to 74 years (4).

CKD has an effect on normal kidney function leading to disturbances in electrolytes such as sodium accumulation and hyperkalemia, metabolic acidosis, which deteriorates the hyperkalemia even more. Reduced ability to maintain an adequate fluid balance leads to fluid retention and fluid overload with the risk of hypertension, heart failure and left ventricular hypertrophy. Disturbances in the renal endocrine function causes reduced vitamin D- and erythropoietin production with subsequent lack of vitamin D, hypocalcemia, osteoporosis and anemia. Reduced ability of renal excretion of water-soluble toxins leads to the accumulation of these toxins/metabolites which causes part of the uremic symptoms along with symptoms related to other disturbances. Symptoms related to uremia include fatigue, weight loss, weakness, decreased mental ability to concentrate, sleeping problem, gastro-intestinal symptom such as nausea, vomiting, gastric ulcer and reduced appetite, symptoms related to fluid overload such as hypertension, edema and dyspnea. Anemia in uremia contribute further to tiredness, reduced physical ability, and deteriorating ischemic heart disease.

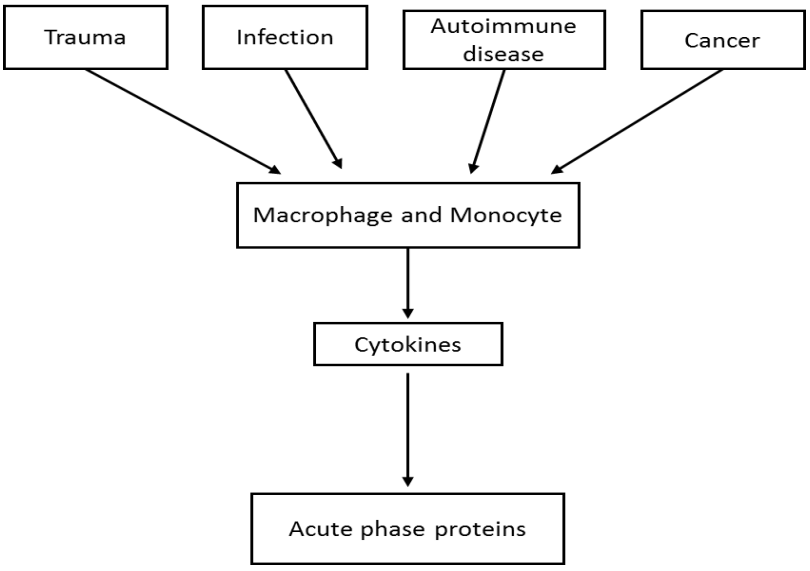
CKD is not only a risk factor for ESRD but also increases the risk for cardiovascular disease and premature death (5). There is a 10 to 20 times higher risk for cardiovascular death for patients treated by hemodialysis (HD) and peritoneal dialysis (PD) compared to the general population at the same age (5). The traditional risk factors such as hypertension, smoking, diabetes mellitus and lack of physical activity are prevalent in CKD patients but these factors cannot explain all of the increased risk of cardiovascular disease in CKD patients. A number of non-traditional risk factors have been identified that may accelerate the process of arteriosclerosis in CKD patients. Some of these factors are inflammation, fluid overload, oxidative stress, anemia, disturbances in calcium and phosphate levels and hyperparathyroidism.

1.2. **Inflammation**

Patients with CKD usually have some degree of chronic low-grade inflammation a condition which is related to increased risk of cardiovascular morbidity and mortality (6). The state of inflammation is linked to premature general and especially vascular aging, a range of metabolic and nutritional derangements including, protein-energy wasting (PEW) as well as acquired immune dysfunction (7-9). Causes for "uremic inflammation" have not been fully elucidated but prior studies have shown an association between chronic inflammation and factors like dietary and lifestyle factors, oxidative stress, dialysis related factors, vascular senescence, periodontal disease, intestinal dysbiosis, depression, immune dysfunction and even kidney function as such with fluid retention (8).

Inflammation is the normal reaction caused by different factors including trauma, infection, malignancy and autoimmune diseases. The body responds to these factors by an inflammatory reaction which starts with migration of monocytes and macrophages to the damaged site. These cells start to produce pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor (TNF) which in turn cause a systemic inflammatory reaction. IL-6 has major effect of enhancing production of acute phase proteins such as C-reactive protein (CRP), α_1-antimycotrypsin, α_1-antitrypsin, orosomucoid and haptoglobin in hepatocyes **(Fig 1)**. Both CRP and IL-6 are strong predictors of cardiovascular mortality among HD patients (10, 11). Chronic inflammation in patients with CKD is very complex because of their impaired defensive response and impaired T cell and neutrophil function (12). The dialysis treatment per se exposes the impaired immune system to high risk of infections, and the contact of blood with the artificial dialysis membrane and uremic solutes also affects the immune system. This combination of impaired immune system and repeated stimulation causes a low degree of inflammation and altered cytokine balance with increased risk of cardiovascular complications. Whereas the IL-10 is considered to be a protective cytokine by suppressing the inflammatory response, cytokines like IL-6 and TNF have shown to be pro-inflammatory and pro-atherogenic (13).

Fig 1: Mechanism of reactions by which inflammatory proteins are produced.



Majority of inflammatory markers are produced in hepatocytes but some of them are procuced at the site of infection/inflammation/tissue damage. Pentraxin 3 (PTX3) is an example of such proteins which is procuced locally in vascular sites by among other endothelial cells (14). Measurements of inflammatory markers are commonly used for detecting or investigating inflammatory reactions or infections and for the clinical follow up of anti-inflammatory treatment or treatment of infectios.

Snaedal el al have shown that CRP has a high variability in HD patients (15). Only 13% of the patients in this cohort had constantly low CRP (< 5mg/L) while 19% hade CRP values greater than 10 mg/l and the other patients had fluctuating values. There was a strong association between the level of CRP and comorbidity. Below some important inflammatory markers used in our studies are described breifly.

1.2.1. **Inflammatory markers**

1.2.1.1. *C-reactive protein (CRP)*

CRP, an ancient highly conserved protein is an acute phase protein. It belongs to the pentraxin family of a class of pattern recognition receptors. CRP participates in the recognition and opsonization of pathogens and even in activation of the complement system. It is produced in the liver stimulated by IL-6. CRP is a part of the innate immune system. It binds to different components in human and bacterial cells such as phosphocholine, C1q and Fc-receptors on anti-bodies. These actions of CRP enhance the phagotisation of bacteria, damaged cells and opsonisation and activation of complement system (16). The plasma concentration of CRP is raised after 4-8 hours in response to infection, trauma or inflammation. CRP has a half time of 19 hours and its concentration decreases fast following an improved clinical condition. This makes CRP a good tool for monitoring infections or inflammatory conditions. CRP along with IL-6 are good predictors of cardiovascular disease sspecially among patients with CKD.

The strong association between inflammation and CVD in CKD patients requires a good and reliable tool for clinical screening and follow up of inflammation in this group of patients (17). CRP levels are stable over time, unaffected by food intake and circadian variation. CRP is widley used and not expensive and can be easily measured. Even though IL-6 has showed to be a superior predictor of cardiovasular outcome, the risk estimate by CRP is close to IL-6 and therefore a good tool for estimating and follow up of inflammation.

1.2.1.2*. Pentraxin3 (PTX3)*

PTX3 together with CRP and serum amyloid P belong to the pentraxin family, a group of acute phase proteins with well preserved structure through the evolution. They are a part of the innate immune system. PTX3 is produced by different cell types, such as macrophages, fibroblasts, endothelial cells, neutrophils and dendritic cells in the vasculature, induced by inflammatory mediators such as LPS, IL-1β and TNF but not IL-6 (14, 18). PTX3 participates in

pathogen recognition and activation of complement system. The role of PTX3 in cardiovascular diseases is debated. Earlier studies have shown that the level of PTX3 is elevated in HD patients with signs of CVD and protein-energy wasting. The levels of PTX3 were reported to be independently associated with all-cause mortality (19). However other studies indicated a cardio-protective role for PTX3 in healthy men (20). In another study, PTX3 was shown to be a predictor of long term all-cause mortality in patients with acute chest pain (21).

1.2.1.3. *Interleukin 6 (IL-6)*

IL-6 is an important cytokine that is involved in several biological activities such as differentiation of B-cells to plasma cells, induction of myeloma and plasmacytoma growth, induction of nerve cell differentiation and production of akute phase proteins. Il-6 is elevated in CKD patients along with CRP and other inflammatory markers. Recent studies indicates that IL-6 is not only a good predictor of cardiovascular disease but also could be an even better prognostic marker than CRP or TNF. Available data indicate that IL-6 is well studied to stratify risk in dialysis and predialysis patients (22).

Sun et al showed in a recent study that IL-6 is the only biomarker that consistently could classify the presence of overt CVD at baseline and predict subsequent mortality over 60 months. After adjustment for age, sex, DM, PEW, smoking, estimated glomerular filtration rate (eGFR) and concomitant analysis of other biomarkers, only high IL-6, high levels of adhesion molecule sVCAM and low serum albumin could classify presence of CVD , and only high white blood cells (WBC) and high IL-6 levels were associated with higher all-cause mortality risk (23).

1.2.1.4*. Orosomucoid (alpha-1-acid glycoprotein)*

Orosomucoid is a small acute phase protein synthesized in hepatocytes. The molecule mass of orosomucoid is about 45 kDa compared to albumin which is 67 kDa. Its production is stimulated by Il-6. The plasma concentration of orosomucoid increases within 24 hours after a tissue damage or an inflammatory reaction. The physiological role of orosomucoid is not fully

understood but it can bind and function as a plasma carrier of many drugs especially weak bases, neutral lipophilic endogenous compounds like steroid hormones and xenobiotics (24, 25). Among basic drugs that bind to orosomucoid are alpha-receptor blocker (prazosin), beta-blockers (metoprolol, timolol, propranolol), analgesics (fentanyl, ketamine, methadone), chlorpromazine, quinidine, and dipyridamole (24, 25). There is an inverse correlation between the levels of orosomucoid and free concentration of drugs with potential clinical consequences such as reduced effectivity or increased risk of adverse side effects depending on the free drug concentration (26).

1.3. **Vitamin D**

In humans, vitamin D is synthesized in 3 steps. The first step is conversion of 7-dehydroxycholesterol to previtamin D_3 in skin under exposure of UVB light (27). This precursor, is in a second step, hydroxylated in the liver to 25-OH-vitamin D_3 (28). In the third step, 25-OH-Vitamin D_3 undergoes a second hydroxylation in the kidneys to produce the 1,25-dihydroxyvitamin D_3, which is the active form of vitamin D. Two major forms of vitamin D are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is the dietary form of vitamin D and could only be supplied via diet or supplementation. The major source of vitamin D_3 is via synthesis from 7-hydroxycholesterol but it could also be obtained via diet or supplementation. The optimal daily dose and the recommended serum level of vitamin D for maintaining musculoskeletal health are suggested to be 800IU and 30 ng/ml (75 nmol/l) respectively (29).

CKD patients are often suffering from vitamin D deficiency due to reduced production of 1,25-dihydroxyvitamin D_3. They need vitamin D supplementation to treat the vitamin D deficiency to avoid clinical complications such as hypocalcemia, osteoporosis and secondary hyperparathyroidism. Alphacalcidol (1α-hydroxyvitamin D) is a vitamin D metabolite administered to CKD patients with vitamin D deficiency on a daily basis. 1α-hydroxyvitamin D can be 25-hydroxylated in liver to the active form of vitamin D and does not require the renal

hydroxylation for activation. It makes it a suitable treatment for vitamin D supplementation in patients with decreased ability of renal hydroxylation of vitamin D. Individual doses for alpha-calcidol in patients in our study ranged between 0.25-0.5 microgram/day. 25-OH-vitamin D_3 is a stable metabolite of vitamin D with a long half-life and considered to be a good measure of vitamin D status in human (30).

1.4. **Drug metabolism**

The concentration of a drug in plasma is determined by processes involved in its pharmacokinetics, i.e., absorption, distribution, metabolism and excretion. Drug metabolism is a process of preparing xenobiotics for excretion. In this process the drugs are converted to more polar compounds which facilitate their excretion. Liver is the main drug metabolizing organ but other organs and tissues such as intestine and kidney might also be involved in this process. The disposition of drugs includes four phases (31, 32):

Phase 0: is the process of drug uptake by the liver from systemic circulation. This process is mediated by transportproteins such as organic anion transporting polypeptides (OATPs) which are located at the basolateral membrane of the hepatocytes. OATPs can also be found in other organs and tissues such as intestine, lung, heart and blood-brain barrier (33).

Phase I: is a process of oxidation of drugs to more polar products to facilitate their excretion. The oxidation reactions are usually catalyzed by members of cytochrome P450 (CYPs) superfamily. These enzymes contain heme proteins and are located in the endoplasmatic reticulum. They use electrons from NADPH to catalyze activation of molecular oxygen to oxidation of substrates as follows:

$$\mathbf{RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+}$$

In this process one oxygen atom is introduced to the substrate RH and the other oxygen atom is reduced to water.

Phase II: is the process of conjugation of drugs per se (e. g. codeine) or metabolites (e.g. morphine) from phase I, which make them even more water soluble. The conjugation reactions are catalyzed by members of the cytosolic sulfotransferase (SULT) and UDP-glycosyltransferase (UGT) superfamilies. SULT catalyzes the transfer of a sulfonyl group to amino or hydroxyl groups of a lipophilic molecule with formation of sulfamate or sulfate conjugates, UGT enzymes catalyze the addition of a glucuronic acid groups to lipophilic substrates such as steroids, drugs, environmental toxins, fat-soluble vitamins and bile acids.

Phase III: is the process of the active secretion of products from phase II into the bile for fecal secretion. This process is mediated by solute carrier transporters (SLC) and members of the ATP-binding cassette (ABC) such as p-glycoprotein (32), which are membrane transporter proteins that use the energy from ATP hydrolysis to translocate hydrophilic substrates across the plasma membrane out of the cytoplasm in a single direction .

1.5. **Cytochrome P450 enzymes**

Presently there are more than 270 different CYP gene families, with ten families comprising 18 subfamilies of which 16 has been mapped in human genomes (34). In humans 60 different CYP enzymes have been identified and families and subfamilies are divided according to the percentage identity in amino acid sequence (34). Enzymes that share ≥ 40% identity are classified into a particular family designed by an Arabic numeral, whereas those sharing ≥ 55% identity are assigned to the same subfamily designed by a letter following the Arabic numeral, e.g. CYP3A, CYP2B. Individual members of a family or subfamily are labelled again by Arabic numeral e. g CYP3A4. These enzymes are localized in the inner membrane of mitochondria or endoplasmatic reticulum and metabolize a wide range of endogenous and exogenous substrates including hormones, cholesterol, vitamin D, bile acids, drugs etc. Enzyme

families CYP1, CYP2 and subfamily CYP3A are important in the metabolism of the endogenous substances while CYP1A2, CYP2C9, CYP2C19 and CYP2D6 are to a major extent drug metabolizing enzymes (35). Factors such as age, sex, and ethnicity, and genetic and environmental factors can influence the CYP activity. The enzymes CYP2D6, CYP2C9 and CYP2C19 are highly polymorphic with different function, described as poor, intermediate, extensive and ultra-rapid metabolizers

1.5.1 CYP2D6

CYP2D6 is one of the most widely investigated CYPs in relation to genetic polymorphism. CYP2D6 metabolizes almost 25% of the drugs used in health care and thus makes it an important drug metabolizing enzyme (36). CYP2D6 metabolizes basic lipophilic drugs such as antidepressants, antipsychotics, antiarrhythmics, antiemetics, opioids and beta-blockers (37).

Genetic mutations such as single nucleotide polymorphism (SNPs), duplications, deletion and/or gene conversion can lead to different phenotypes such as poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM) and ultra-rapid metabolizer (37). The clinical consequence of different phenotypes depends on whether the concentration of an active parent drug or metabolite increases or decreases in plasma due to increased or decreased metabolism (37) or if there are more than one enzyme responsible for the metabolism of a certain drug (38).

Another important effect of CYP2D6 genetic polymorphism on drug metabolism involves the bioavailability. EMs show high first pass extraction leading to low bioavailability while PMs have low first pass extraction which causes high bioavailability of the drug.

The prevalence of PM and EM metabolizers varies among different populations. Four major mutant alleles termed CYP2D6*3–*6 are associated with the PM genotype/ phenotype in Caucasians. The CYP2D6*4 allele has a frequency of 22% in Swedish Caucasians and accounts

for >75% of the detrimental alleles in this population (39). As this allele is almost absent in Chinese, the prevalence of PMs among Chines is low: 1% compared with 6% in Caucasians (40). The alleles CYP2D6*1 and *2 comprise the EM phenotype, and its duplication/multiplication leads to higher metabolic activity. Gene duplication shows a European-African, north−south gradient, with a prevalence of 1–2% in Swedish Caucasians and 29% in Ethiopians (41). The polymorphisms are named according to The Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se).

1.5.2 **CYP3A**

CYP3A enzymes comprise a group of four enzymes including CYP3A4, CYP3A5, CYP3A7 and CYP3A43 which have similar amino acid sequences but with different substrate specificities. These enzymes have large active sites and very broad substrate specificity.

Due to high involvement of CYP3A families in drug metabolism it is important to identify markers of CYP activity for measuring its activity in humans. Some of the clinical markers presented in literature are:

- Measuring the plasma clearance of midazolam by calculating area under the curve (AUC) after oral or intravenous administration (42).
- Measuring the metabolic ratio for quinine (quinine/3-OH-quinine) after an oral administration (43).
- Measuring the plasma level of 4β-hydroxycholesterol in plasma (44).
- Measuring the ratio of 4β-hydroxycholesterol/cholesterol in plasma (45).

1.5.2.1 *CYP3A4*

CYP3A4 is the major adult-specific isoform in the CYP3A subfamily. It has two large active sites with a broad substrate specificity and is expressed abundantly both in the human liver and the intestine but it is also present in other organs. CYP3A4 is the most important drug-

metabolizing enzyme, which metabolizes more than 50% of the drugs currently used in health care (46, 47). Several nuclear receptors such as PXR (pregnane X-receptor), CAR (constitutive androstane receptor), VDR (vitamin D receptor) and glucocorticoid receptors mediate the transcriptional regulation of CYP3A4, which may explain the wide inter-individual variation in expression and activity (48). More than 40 different alleles have been identified (http://www.cypalleles.ki.se/cyp3a4.htm) and among them CYP3A4*20 and CYP3A4*26 have been shown to be non-functional.

1.5.2.2 *CYP3A5*

CYP3A5 is predominantly expressed in liver and intestine but the expression of this allele is higher in kidney compared to CYP3A4. It has structural similarity to CYP3A4 by sharing 84% of their amino acid sequences (49). CYP3A5 has shown a marked genetic polymorphism with both inter-individual and inter-ethnic variation in expression (50). Diczfalucy et al. showed marked differences in the expression of CYP3A5 between black Tanzanians (74%), Asian Koreans (33%) and Caucasian Swedish subjects (13%) (50). The common cause of genetic variation like many other CYPs is SNPs e.g. CYP3A5*3 and CYP3A5*6 causing truncated and non-functional enzymes (51).

1.5.2.3 *CYP3A7*

CYP3A7 is the pre-dominant CYP enzyme in neonate's liver and intestine accounting for 50% of the hepatic CYP content in neonates (52). It shares 88% amino acid sequence with CYP3A4. This enzyme will be replaced by CYP3A4 during first year of life (52).

1.5.2.4 *CYP3A43*

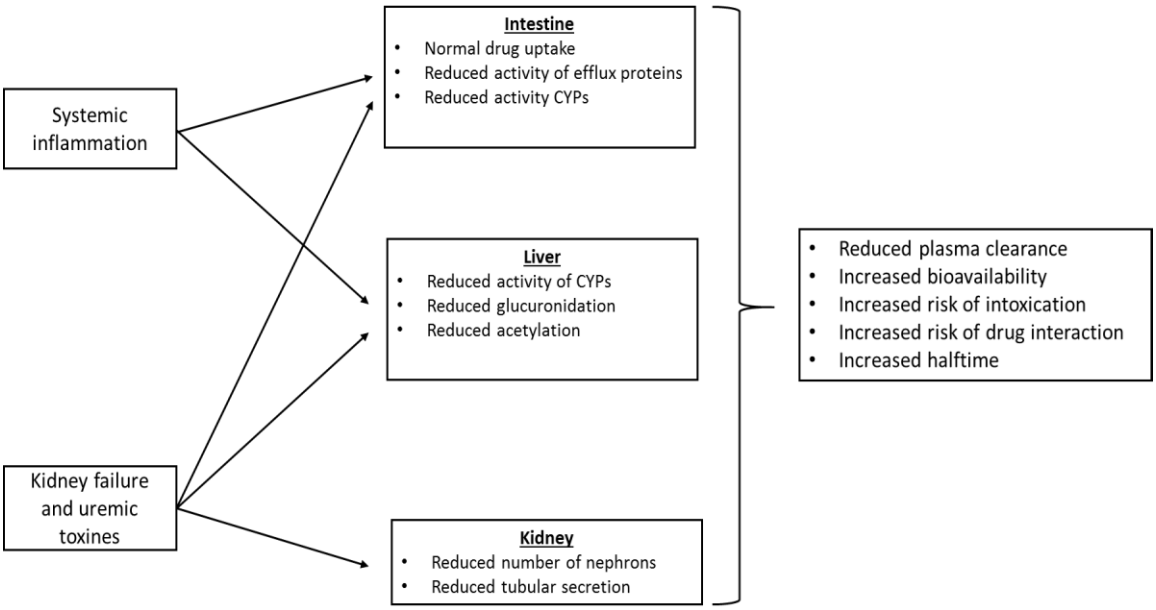
CYP3A43 is expressed to a lower extent than CYP3A4 in human liver and intestine and therefore it has minor role in drug metabolism (53). It is has however higher levels of

extrahepatic expression with highest levels in the brain (54). The amino-acid sequence of CYP3A43 is 75% identical to CYP3A4 and CYP3A5 (55).

1.6. **Pharmacokinetics of drugs in kidney failure**

All the aspects of pharmacokinetics of drugs are more or less affected in kidney failure, which results in increased concentration and thereby the bioavailability and disposition of the drugs. This can increase the risk of drug intoxication and drug-drug interaction. A summary of pharmacokinetics of drugs is shown in **Figure 2**.

Figure 2: Pharmacokinetics of drugs in kidney failure.



1.6.1 **Drug absorption**

The intestinal uptake of orally administered drugs into the enterocytes are mediated by uptake transporters such as organic anion transporting polypeptide (OATP). The absorbed drug is metabolized by intestinal CYPs, CYP3A in particular. The drug or its metabolite will then be either actively transported back into the intestinal lumen by transporters such as P-glycoprotein (P-gp) and multidrug resistance–related protein 2 (MRP2) or transferred into the portal vein by diffusion or active transport mediated by MRP1 or MRP3 (56).

Earlier studies have indicated that the activity of drug metabolizing enzyme in intestine is downregulated in CKD patients (57, 58). At the same time the activity of P-gp as well as protein expression of P-gp, MRP2, and MRP3 are significantly decreased CKD patients but there is no change in their mRNA expression and uptake transporters OATP2 and OATP3 were found to be unaffected by kidney failure (59) which means that the uptake of the drug is unaffected but the efflux of the drug is reduced. Patients with CKD have even a chronic low degree inflammation and inflammation per se may alter the activity of drug metabolizing enzyme and transporters (60). Adding these factors with reduced drug metabolism (due to reduced CYP activity) leads to even more increased risk of drug accumulation.

There are also several other factors in the intestine of a uremic patient that could affect the absorption of a drug and the activity of drug metabolizing enzymes (61). Uremic patients have disturbed bacterial flora leading to increased risk of infection and exposing the uremic patient for bacterial toxins with subsequent inflammation. Factors such as reduced intestinal motility, intestinal edema, metabolic acidosis, fermentation of urea to ammonia by intestinal bacteria leading to altered pH of the intestine, and frequent use of antibiotics, are factors that could contribute to disturb the bacterial flora in intestine (61).

1.6.2 **Distribution**

The volume of distribution (V_d) of a drug could change in CKD patients depending on the degree of plasma and/tissue protein binding. CKD patients suffer from hypoalbuminemia due to among others albuminuria and malnutrition. Uremia can also change albumin binding sites and reduce the affinity for acidic drugs. Furthermore, the protein binding of acidic drugs to albumin is reduced because CKD patients are taking several medications which together with uremic toxins are competing for binding sites on albumin. This increases the concentration of the unbound fraction of drugs causing V_d to increase (62). The free unbound fraction of the drug increases the risk of drug intoxication but on the other hand it will be more available for excretion from plasma.

Basic drugs are mostly bound to nonalbumin plasma proteins such as orosomucoid, which is an acute-phase protein whose plasma concentrations have been demonstrated to be elevated in kidney disease due to chronic inflammation. Higher degree of plasma protein binding will in turn decrease the V_d (62, 63).

1.6.3 **Drug metabolism in CKD**

Several studies have shown that the activity of drug metabolizing enzymes are reduced in CDK patients (64). Hemodialysis improves the function of Cyp1A, Cyp2C, and Cyp3A (65). This indicates that uremic factors may be causing decreases in the protein expression and activity of these enzymes. The presence of chronic low degree inflammation in CKD patients is another factor that may contribute to reduced metabolic capacity of both liver and intestine. Reduced activity of the drug metabolizing enzymes increases the risk of drug accumulation, drug-drug interaction and intoxication.

Even the phase II reactions such as glucuronidation and acetylation are reduced in CKD patients which are demonstrated by reduces metabolic capacity of morphine (66) and procainamide (67) respectively in CKD patients.

1.6.4 **Renal excretion**

The ability of elimination of renally excreted drugs are significantly reduced in CKD patients due to reduced number of functional units (nephrons) and reduced tubular secretion of several drug with subsequent accumulation of their plasma concentration despite administration of normal dose (68).

2. **Objectives**

2.1. **Study 1**

To study the influence of three factors on drug disposition: genetic polymorphism, impaired renal excretion of drug metabolites, and the possible elimination by hemodialysis (HD), using codeine as a model drug.

2.2. **Study 2**

To investigate the impact of persistent inflammation in hemodialysis (HD) patients on the pharmacokinetics of alprazolam, a cytochrome P450 (CYP) 3A4 substrate, and its metabolites and the role of HD and the impact of persistent inflammation in this clinical context.

2.3. **Study 3**

To investigate the association of CYP3A4-mediated metabolism of quinine, with inflammatory biomarkers in patients undergoing maintenance hemodialysis (HD).

2.4. **Study 4**

To investigate the possible association between vitamin D supplementation and drug metabolism, using quinine as a test drug.

3. Methods

3.1. **Study populations**

3.1.1 **Study 1**

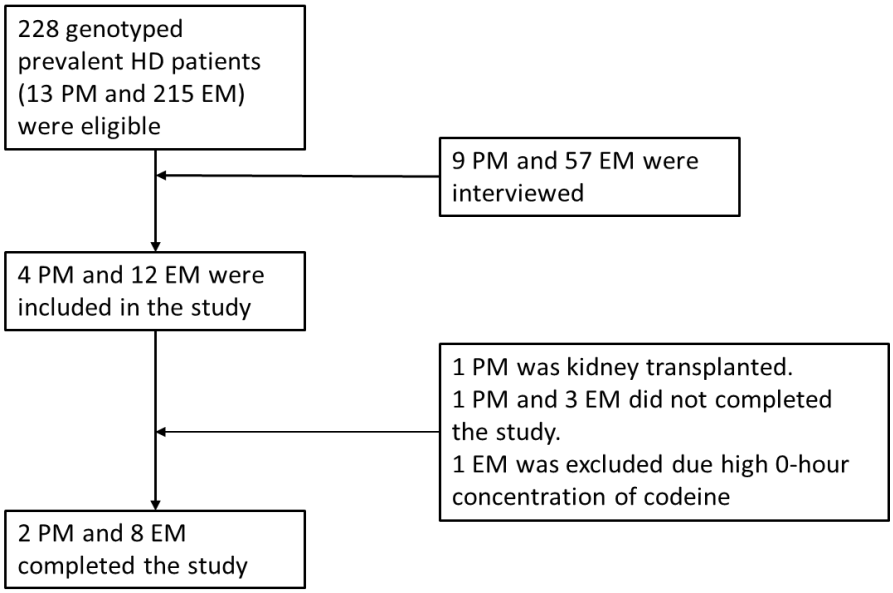
This study was performed at the Karolinska University Hospital, Huddinge, Sweden and its satellite HD units in the Stockholm area. Patients who agreed to participate gave their written informed consents on the basis of verbal and written information. The Ethics Committee of the Karolinska Institutet, Huddinge approved the study and informed consent was obtained from all patients. Initially, 228 prevalent adult HD patients were genotyped regarding CYP2D6. Thirteen patients (5.7 %) had two variant alleles representative of poor metabolizers (PM). The remaining patients were considered as extensive metabolizers (EM). A selected group of the patients were able to participate in a subsequent pharmacokinetic study (two PMs and 9 EMs) **(Figure 3)**. The general characteristics of the 11 patients participating in this pharmacokinetic study are presented in **Table 2**.

Table 2: General characteristics of the nine EMs and two PMs included in the study

	EM (n=9)[a]	PM#1	PM#2
Age (years)	66 (47-76)	77	55
Female/male (n)	3 / 6	Male	Male
Time on dialysis (months)	57 ± 6	60	12
BMI (kg/m^2)	26 ± 4	32	30
Albumin (g/l)	36 ± 4	39	40
Kt/v	1.45±0.19	1.35	1.44
Urea reduction rate (%)	75±0.1	66	56
Diabetes mellitus (n)	2	Yes	Yes
Cardiovascular disease (n)	3	Yes	Yes
Primary kidney disease	DM: 2 GN: 4 Vasculitis: 1 Unknown: 2	DM	PCKD

Abbreviations: BMI, body mass index; DM, diabetes mellitus; GN, glomerulonephritis; Kt/V, urea dialyzer clearance K multiplied by treatment time t and divided by urea distribution volume V. [a] *One EM was excluded due to high 0-hour concentration of codeine.*

Figure 3: Flow chart for the participants in the study 1.



3.1.2 **Study 2**

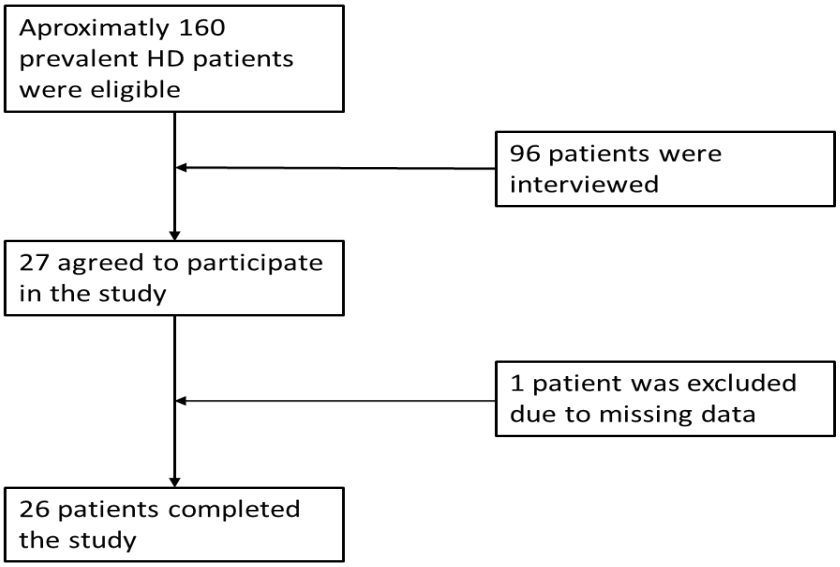
This study was performed at the Karolinska University Hospital, Huddinge, Sweden and its satellite HD units in the Stockholm area. The Ethics Committee of Karolinska Institutet, Huddinge approved the study. Twenty-seven prevalent HD patients agreed to participate in our study and all participants gave their written informed consent on the basis of verbal and written information. Results from one patient were not included in the final calculations due to some missing data **(Figure 4)**. The general characteristics of the remaining 27 patients participating in this pharmacokinetic study are presented in **Table 3**. All the patients had three HD sessions per week (3.5-5 hours/session) using low-flux polysulfone membrane dialyzers.

Table 3: General characteristics of 27 patients who participated in study 2.

Age (years)	63 ± 15
Males (%)	68
Dialysis vintage (months)	32 (7-156)
BMI (kg/m^2)	25 ± 4
S-albumin (g/L)	37 ± 4
CRP (mg/L)	4.9 (0.75-24)
Hemoglobin (g/L)	117 ± 14
Kt/v	1.4 ± 0.3
Urea reduction rate (%)	74 ± 5
Cardiovascular disease n (%)	31
Primary Kidney Disease (n)	Diabetes nephropathy 7 Glomerulonephritis 4 Hypertension 6 Unknown origin 3 Vasculitis 1 Pyelonephritis 1 PCKD 3 Myeloma 1 Juvenile nephritis 1

Abbreviations: BMI, body mass index; Kt/V, urea dialyzer clearance K multiplied by treatment time t and divided by urea distribution volume V; PCKD, polycystic kidney disease.

Figure 4: Flow chart for the participants in the study 2.



3.1.3 **Study 3**

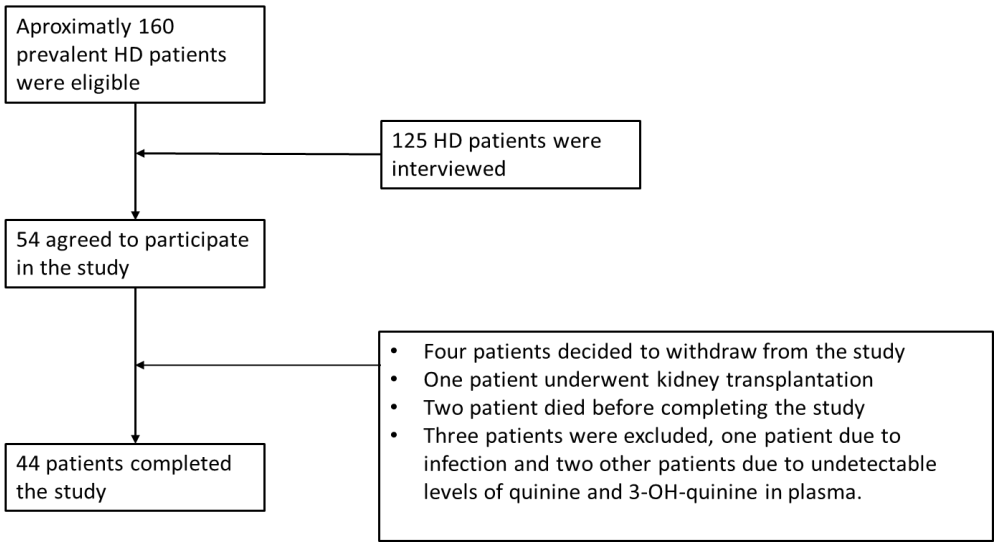
Fifty-four prevalent HD patients with no or minimal residual renal function agreed to participate in the study and gave their written informed consent following verbal and written information. Ten patients did not complete the study (**Figure 5**). Baseline characteristics of the patients are shown in **Table 4**. All patients underwent three HD sessions per week (3.5-5.0 hours/session) using high-flux polysulfone membrane dialyzers. The study was performed at the Karolinska University Hospital, Stockholm, Sweden. The Ethics Committee of Karolinska Institutet, Stockholm approved the study. All the patients were included in the study during the four months period between November 2011 and Mars 2012. They were followed up during five weeks from the initiation of the study and until the study was completed.

Table 4: General characteristics of 44 patients who completed study 3.

Age, years	71 (61 - 77)
Female/male, n	11/33
Dialysis vintage, months	36 (20 - 72)
Body mass index, kg/m²	25.0 (23.0 - 29.5)
Diabetes mellitus, n	23
Cardiovascular disease, n	30
Primary kidney disease, n	
Diabetes mellitus	12
Chronic glomerulonephritis	5
Nephrosclerosis	7
Polycystic kidney disease	4
Vasculitis	2
Other or unknown disease	14
Medications, n	
Beta-blockers	25
ACEi/ARBs	9
Statins	15

Data presented as number of patients (n), or as median and interquartile range (IQR). Abbreviations: ACEi: angiotensin converting enzyme inhibitor, ARBs; angiotensin receptor blockers.

Figure 5: Flow chart for the participants in the study 3.



3.1.4 **Study 4**

This study was performed at the Karolinska University Hospital, Stockholm. The Ethics Committee of Karolinska Institutet, Stockholm approved the study. Forty-four prevalent HD-patients who participated in a prior study 3 were invited to participate in this study. The idea of this selection was to have a group with low 25-OH-vitamin D_3 levels that could be its own control group. Fourteen HD patients with low levels of 25-OH-vitamin D_3 agreed to participate in the study but only 8 HD patients completed the study. The flow chart for participants in this study is shown in **Figure 6**. Baseline characteristics of the patients who completed the study are shown in **Table 5**.

Figure 6: Flow chart for the participants in the study 4.

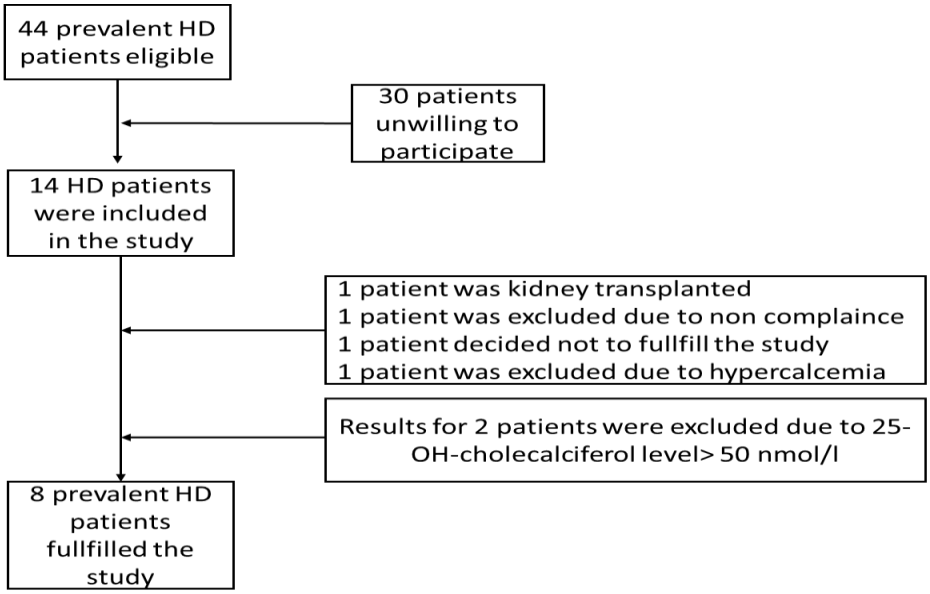


Table 5: Baseline characteristics of 8 HD patients who completed study 4.

Age, years	**76 (52-84)**
Female/male, n	2 / 6
Dialysis vintage, months	43 (7-392)
Body mass index, kg/m^2	28 (21-33)
Diabetes mellitus, n	6
Cardiovascular disease, n	7
Primary kidney disease, n	
Diabetes mellitus	3
Vasculitis	1
Other or unknown disease	4
Medications,	
Alphacalciferol at study start n	7

Values are given as median and range or numbers (n).

3.2 **Study procedure**

3.2.1 **Study 1**

Patients were given a single oral dose of 50 mg codeine phosphate (Kodein Recip® 2 x 25 mg) at 8A.M. following an overnight fast. Blood samples (10 ml) were drawn via an indwelling intravenous catheter into heparinized Vacutainer® tubes before codeine intake and after 2, 4, 6, 8, and 24 (concurrent with the beginning of the hemodialysis session), and 28 h (concurrent with the end of the 4-h hemodialysis session). Samples were centrifuged and plasma separated and stored frozen at −20°C until analysis.

3.2.2 **Study 2**

Each patient was given 1 mg alprazolam (Xanor, 1 mg; Pfizer, New York, NY) orally in the evening before the day of dialysis. None of the patients were being treated with CYP3A4 inhibitors or inducers. Peripheral blood samples (10 ml) were collected into EDTA-containing tubes at 10, 34 (start of HD) and 38h (end of HD) after alprazolam intake. The plasma was separated and stored frozen at -20°C until analyzed. High sensitive CRP (hsCRP) was followed once e week during 8 weeks prior to the drug test. Median value of hsCRP from the last 5 week was calculated to measure the degree of inflammation.

3.2.3 **Study 3**

Study design and time points for blood samples taken during the study are shown in **Table 6.**

Table 6: Study design, patient flow and samples taken during study 3.

Week 1 n= 54 [a]	**Week 2** n= 50	**Week 3** n= 49	**Week 4** n= 47	**Week 5** (n: 44)	
				Day 1	**Day 2**
• Inclusion • hsCRP, PTX3, orosomucoid	• hsCRP, PTX3, orosomucoid	• hsCRP, PTX3, orosomucoid	• hsCRP, PTX3, orosomucoid	• Administration of 100 mg quinine	• hsCRP, PTX3, IL-6 and orosomucoid • Hb, P-Iron, P-albumin, pro-BNP and PTH • 4β-OH-Cholesterol and P-Cholesterol • Concentration of quinine and 3-OH-quinine

[a] At screening; hsCRP (high sensitive CRP), PTX3 (pentraxin 3), IL-6 (interleukin-6), Hemoglobin (Hb), pro-BNP (pro-brain natriuretic hormone).

Each patient was given 100 mg quinine (Kinin®100 mg) orally in the evening before the day of a regular dialysis day. Samples of 10 ml peripheral blood were drawn into EDTA containing tubes at the beginning of the dialysis session. Plasma was separated and stored frozen at -80°C until analysis. The concentration of quinine and its metabolite 3-OH-quinine was measured in the blood sample collected 12 hours after drug intake at the beginning of the dialysis. The ratios of quinine and 3-OH-quinine (43) and 4β-OH-cholesterol/cholesterol (44) were used as markers for CYP3A4 activity. The levels of hsCRP, orosomucoid, and pentraxin 3 (PTX3) were measured weekly before dialysis sessions during the four weeks prior to the drug test **(Table 6)**. Measurements were repeated in plasma samples collected 12 hours after drug intake concomitantly with measurements of 4β-OH-cholesterol, cholesterol, hemoglobin, albumin, iron, pro-BNP, parathyroid hormone (PTH) and interleukin-6 (IL-6) (**Table 6)**.

3.2.4 **Study 4**

Blood samples were collected after enrolment for measurement of hemoglobin, iron, NT-pro-BNP, albumin, 25-OH-vitamin D_3, 1,25-dihydroxyvitamin D_3, 4β-OH-cholesterol, cholesterol and inflammatory markers such as high sensitive CRP (hsCRP) and orosomucoid at basal and at the end of the study. Each patient started the supplementation of vitamin D by taking a daily dose of 800 IU 25-OH-vitamin D_3 (Divisun® 800IU). All eight patients except one were treated with 1α-hydroxyvitamin D (Etalpha®) at basal. 1α-hydroxyvitamin D is a vitamin D analogue, which is clinically used for treatment hyperparathyroidism and deficiency of active vitamin D in HD patients as part of general recommendation for treatment of these patients. The patients in this study maintained their treatment with 1α-hydroxyvitamin D along with 25-OH-vitamin D_3 supplementation. Concentrations of 25-OH-vitamin D_3 were followed once per month and the doses of 25-OH-vitamin D_3 supplementation were adjusted accordingly. The aim of treatment was 25-OH-vitamin D_3 concentration > 75 nmol/L or 30 ng/mL (conversion factor for 25-OH-vitamin D_3 is 1 ng/mL = 2.496 nmol/L). The level of >75 nmol/L is considered to be adequate according to KDOQI guidelines (69). The range for Interindividual doses for substitution of 25-OH-vitamin D_3 in this study was between 800-1600 IU and the duration of treatment ranged from 3 to 21 months.

After reaching the target plasma level of 75 nmol/L of 25-OH-vitamin D_3, CYP3A4 activity was evaluated in each patient in an identical way as in study 3. The patients were given a test dose of 100 mg quinine (Kinin®100 mg) orally in the evening before the day of a regular dialysis day. Samples of 10 ml peripheral blood were drawn into EDTA containing tubes at the beginning of the dialysis session. Plasma was separated and stored frozen at -80°C until analysis. The concentration of quinine and its metabolite 3-OH-quinine were measured in the blood sample collected 12 hours after drug intake at the beginning of the dialysis. As in study 3 the ratios of quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol were used as markers of CYP3A4 activity.

3.3 **Biochemical analysis**

3.3.1 **Study 1**

Codeine metabolites C6G, M3G, and M6G were quantitated by liquid chromatography−mass spectrometry (LC−MS) with atmospheric pressure ionization−electrospray (API-ES) detection, as described by Svensson et al. (70). A 50-µL sample was prepared for analysis by mixing it with 200 µL internal standard (IS) solution (containing M3G- d_3, 1 µg/mL; codeine-d_3 1 µg/mL; and 6-AM-d_3 [acetylemorphine], 100 ng/mL in ammonium acetate buffer) and 500 µL ammonium sulfate buffer. The mixed sample was brought on cartridge and then eluted with 500 µL of 20% acetonitrile in 25 mmol/L formic acid. The eluate was centrifuged in a vacuum for 15 min to evaporate the acetonitrile, which resulted in a 60% reduction in sample volume, and 100 µL was transferred to a 200-µL vial.

The LC-MS analysis was performed using an Agilent 1100 LC-MS instrument (model 3.01.99) with the ESI interface. The instrument was controlled by the Chemstation software (Version B 01.03) and was equipped with a dual LC pump, degasser, column thermostat, and an autosampler. The gradient mixing chamber was bypassed to reduce the gradient delay time. Sample extract (5 µL) was injected onto a column. And the column was eluted by buffer A (1% acetonitrile) and buffer B (90% acetonitrile), both with 25 mmol/L of formic acid. The identification of analytes in unknown samples was based on chromatography and quantification was performed using calibration graphs constructed from peak- height ratios between analytes and the respective IS. Values used for the glucuronides were calculated to represent the free form of the compounds.

The lower limit of quantification for these three substances was 1 nM. Unconjugated codeine and morphine could be quantitated in some but not all patients, and we decided to report only the concentrations of glucuronides, where detailed kinetics could be evaluated.

3.3.1.1 *CYP2D6 genotyping by pyrosequencing*

For analysis of the CYP2D6*3, *4, and *6 alleles (rs35742686, rs3892097 and rs5030655, respectively), primers were as reported by Zackrisson and Lindblom (71). All oligonucleotides were synthesized by Thermo Electron Corporation (Waltham, MA, USA). The pyrosequencing reaction was performed on a PSQ96™MA instrument from Biotage AB (Uppsala, Sweden), as described previously (72).

3.3.2 **Study 2**

The concentrations of conjugated and unconjugated alprazolam and its metabolites α- and 4-hydroxyalprazolam were quantified in plasma by LC-MS (liquid chromatography–mass spectrometry) as described by Allqvist et al. (73). Total concentrations (unconjugated + conjugated) of alprazolam and its metabolites were determined after hydrolysis with β-glucuronidase (from Escherichia coli K12; Roche, Basel, Switzerland). In brief, 400 µl of plasma was mixed with 100 µl β-glucuronidase solution (prepared by mixing 1.5 ml β-glucuronidase with 5 ml of 1.0 M potassium phosphate buffer pH 6.0) and incubated at 37°C for 15 min. The hydrolyzed compounds were quantitated as described previously for unconjugated alprazolam and metabolites (70). All samples from each patient were analyzed in duplicate, and the average of these two values was calculated. The area under the curve (AUC) and half-life (t½) were calculated for alprazolam and its metabolites using the 10- and 34-h concentrations on a semi-logarithmic graph. The 0-h concentration could also be extrapolated from this graph. CYP3A4 activity was estimated as the ratio of unconjugated alprazolam to 4hydroxyalprazolam and expressed as AUC10–34h. The basal (baseline) level of alpha 1-acid glycoprotein (orosomucoid) was measured, and the hsCRP level was measured weekly during the 8 weeks prior to the intake of the test drug to estimate the degree of inflammation. The median value for the last five hsCRP measurements was calculated. Plasma alpha 1-acid glycoprotein, cholesterol and hsCRP levels were measured by validated routine methods used by the accredited clinical chemistry laboratory at Karolinska University Hospital, Huddinge. Plasma 4β-hydroxycholesterol was analyzed according to Bodin et al. (74). After primary preparation

samples were separated, using a solid-phase extraction column. Analytes were measured by gas chromatography-mass spectrometry.

3.3.3 **Study 3**

The concentrations of quinine and its metabolite 3-OH-quinine were determined by high-performance liquid chromatography with tandem mass spectrometric detection (UPLC-MS/MS) following sample separation by protein precipitation with acetonitrile containing internal standards as described by Björkhem-Bergman et al. (43). A 100 µL volume of the sample was protein precipitated with 200 µL of the internal standard solution. The extract was injected into the UPLC-MS/MS system. Separation of the analytes was achieved on an Acquity UPLC BEH C18-column (2.1 x 50 mm 1.7 µm), using gradient run with mobile phase A (11 mM ammonium formate) and mobile phase B (0.1% formic acid in acetonitrile). The analytes were detected using a Micromass Quattro Primer XE mass spectrometer operating in positive electrospray ionization (ESI) mode utilizing selected reaction monitoring (SRM) for the transitions 325→160 m/z for quinine and 341→160 m/z for 3-OH-quinine.

IL-6 was analyzed in serum by an immunometric assay on an Immulite 1000 Analyzer (Siemens Healthcare, Los Angeles, CA, USA) according to the instructions of the manufacturers. PTX3 was analyzed in EDTA plasma with sandwich ELISA from R&D systems (Abingdon, UK). Cholesterol was determined on a Roche/Hitachi Modular instrument using a commercial enzymatic method (Cholesterol CHOD-PAPP, Roche Diagnostics, GmbH, Mannheim, Germany). The between-day variation was 1.3% at 5 mmol/L. Orosomucoid, hemoglobin, iron, pro-BNP, albumin, and hsCRP in plasma were measured by validated routine methods used by the accredited clinical chemistry laboratory at Karolinska University Hospital, Stockholm. Plasma 4β-OH-cholesterol was determined by gas chromatography-mass spectrometry as described in study 2.

3.3.4 **Study 4**

4β-OH-cholesterol, cholesterol, quinine and its metabolite 3-OH-quinine were measured as described in study 3. Levels of hemoglobin, iron, NT-pro-BNP, albumin, 25-OH-vitamin D_3, 1,25-dihydroxyvitamin D_3 and inflammatory markers such as high sensitive CRP (hsCRP) and orosomucoid were measured by validated routine methods used by the accredited clinical chemistry laboratory at Karolinska University Hospital, Huddinge.

3.4 **Statistical analysis**

3.4.1 **Study 1**

All variables were expressed as mean ± SD, unless otherwise indicated. Statistical significance was set at the level of $p< 0.05$. Comparisons between two groups were assessed with the nonparametric two-tailed Wilcoxon signed-rank test for matched pairs. All statistical analyses were performed using JMP software (SAS Institute Inc) version 7.0.1.

3.4.2 **Study 2**

All variables were expressed as the mean ± standard deviation (SD), median (range) or percentage, unless otherwise indicated. Statistical significance was set at the level of $P<0.05$. Correlations between two groups of values were assessed using the nonparametric Spearman rank's test or paired student t test. Analyses were performed using the Prism 5 software program (GraphPad Software, San Diego, CA).

3.4.3 **Study 3**

All variables are expressed as mean ± SD or as median (25^{th} and 75^{th} percentile), unless otherwise indicated. Statistical significance was set at the level of $p\leq0.05$. Comparisons

between two groups were assessed with the nonparametric Wilcoxon test. Fischer´s exact test or chi-square test was used for categorical variables. Non-parametric Spearman's rank correlation analysis was used to determine associations between various variables. Determinants of ratio quinine/3-0H-quinine were explored using linear multivariate regression analysis. All statistical analyses were performed using statistical software SAS version 9.4 (SAS Campus Drive, Cary, NC, USA).

3.4.4 **Study 4**

All variables are expressed as mean ± SD, unless otherwise indicated. Statistical significance was set at the level of $p<0.05$. Comparisons between two groups were assessed with student t-test. Non-parametric Spearman's rank correlation analysis was used to determine associations between various variables. All statistical analyses were performed using statistical software GraphPad Prism 5.

3.5 **Ethical approvals**

Regional Ethical Review Board in Stockholm approved all the studies in this thesis.

4. Results and Discussions

4.1.1 Results Study 1

Thirteen out of the 228 patients (5.7%) were genotypically PMs on the basis of their CYP2D6*3, *4, and *6 alleles. Eleven patients completed the pharmacokinetic study with codeine. Of those, two PMs had the CYP2D6*4/*4 genotype, four EMs hade the CYP2D6*1/*4 genotype, and five EMs had the CYP2D6*1/*1 genotype. Results from one EM patient were not included in the study because of high 0-h concentrations of M3G, M6G, and C6G, indicating that the patient had either taken another codeine-containing drug or taken the test drug

earlier than the time indicated in the protocol. Concentrations of C6G were similar in the remaining eight EMs and the two PMs **(Fig. 7, Table 7)**. Plasma concentrations of C6G, M3G, and M6G, which are normally excreted by the kidneys, increased during the early phase and remained unchanged for 24 h until the start of HD **(Fig. 7)**. Two hours after codeine intake, the mean concentration of M3G was 210 nM in EM compared with 3.5 nM in PM **(Fig. 7)**. M6G was not detectable (<1 nM) in one of the remaining eight EMs and in either PM. The plasma concentrations of both M3G and M6G were thus much lower in PMs compared with EMs **(Table 7)**, indicating less formation of morphine in PMs. Earlier, larger studies showed a difference in metabolic capacity between CYP2D6 homozygote and heterozygote genotypes (41), but in this small group, there was no significant difference in kinetics. During HD, there was a rapid decrease of plasma concentrations of all three glucuronides, but they could still be quantified in most patients at the end of HD **(Table 7)**.

Figure 7: Plasma concentrations (nM) of codeine-6-glucuronide (C6G), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in 8 EMs (broken lines) and 2 PMs (unbroken lines). BHD: before hemodialysis, AHD: after hemodialysis.

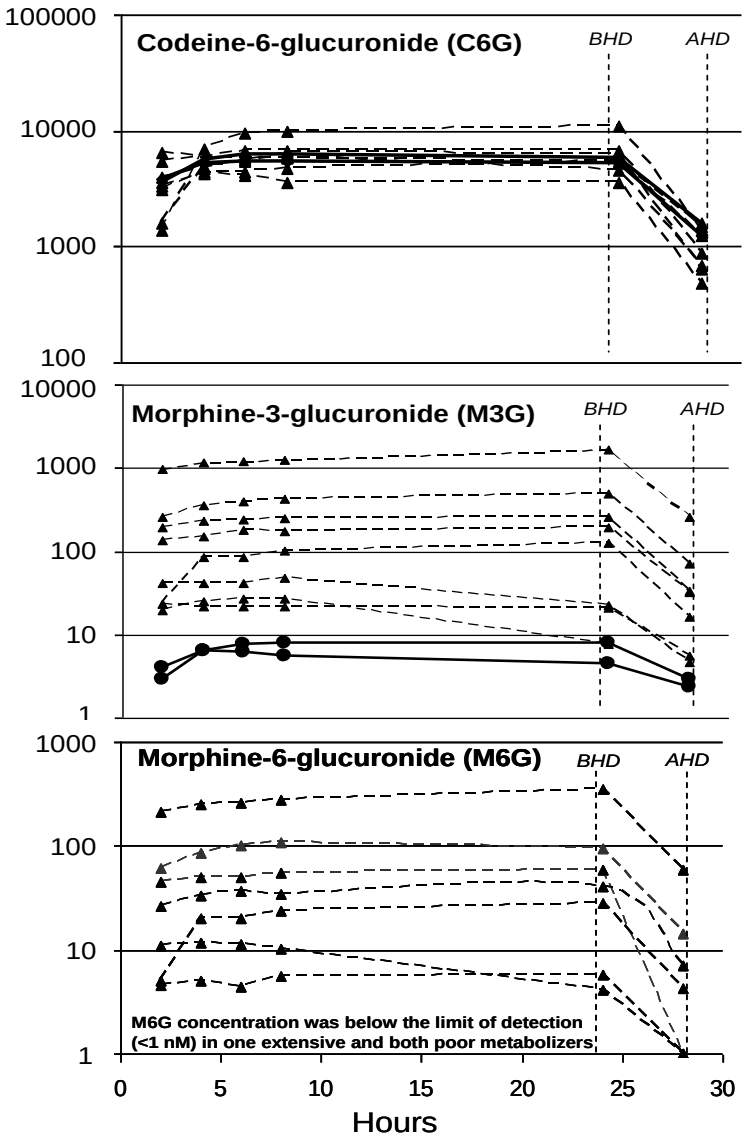


Table 7: Mean plasma concentrations (nM) of codeine metabolites (±SD) in 8 EMs before and after hemodialysis. The individual values in the two PMs are given and no statistics are calculated as only two PM participated (N.D. = not determined)

Metabolite	**Genotype**	**Before HD (24h)**	**After HD (28h)**	**P-value**
C6G	**EM**	5591±2673	889±443	0.008
	PM1	5278	1266	N.D.
	PM2	5851	1605	N.D.
M3G	**EM**	348±555	53±86	0.008
	PM1	4,4	2,4	N.D.
	PM2	8,0	2,9	N.D.
M6G	**EM**	74±118 [a]	11±20	0.01
	PM1	Not quantifiable (< 1nM)		
	PM2			

Codeine-6-glucuronide (C6G), Morphine-3-glucuronide (M3G), Morphine-6-glucuronide (M6G). [a] *One out of the 8 EMs had M6G concentrations < 1nM and is here given the level of 1nM.* ***P*** *as assessed by the Wilcoxon Sign-Rank test for matched pairs.*

4.1.2 Discussion Study 1

The frequency of PMs' genotype/phenotype of CYP2D6 in a healthy Swedish population is 6–7% (40), and of 228 HD patients studied in our units, 13 (5.7%) were genotypically PMs. Thus, the prevalence of PMs does not seem to differ between Swedish ESRD patients and healthy Swedish individuals. CYP2D6 gene duplication was not investigated, as it was less likely to find any individual with gene duplication in nine EMs in our study because the frequency of individuals with duplicated/multi-duplicated gene in Swedish Caucasians is about 1–2% (75). Codeine is mainly metabolized by glucuronidation and to a minor extent by CYP2D6 to active morphine in the liver. Only approximately 3% is excreted unchanged by the kidneys. Plasma half-life of codeine is about 3 h in healthy individuals (76, 77). An earlier study on codeine showed that clearance of codeine metabolites was significantly reduced and the elimination half-life of codeine was longer in HD patients compared with healthy individuals, but there was no clinically significant difference in pharmacodynamics (78). In that study, however, patients were not genotyped, and the impact of hemodialysis on codeine elimination was not studied. In our study, we found that plasma concentrations of glucuronides increased initially and then remained high during the 24 h until HD started **(Figure 7)**. We showed that M3G and M6G concentrations are markedly lower in PM HD patients compared with EMs. In EMs, the glucuronidated metabolites M3G and M6G reached high plasma concentrations compared with those in normal individuals (76). This finding is in accordance with earlier studies that showed retention of glucuronidated metabolites of morphine and oxazepam to high concentrations in patients with renal function impairment (78, 79). In our study, plasma concentrations of glucuronidated metabolites M3G and M6G remained almost unchanged for 24 h after administration of codeine or even tended to increase until the start of the HD session **(Figure 7)**. Although there was a significant reduction in concentrations of the three glucuronides by HD, the codeine metabolites were not totally eliminated from plasma. M6G was present in plasma during the interdialytic phase, which may lead to an analgetic effect during this time and possibly the risk of side effects, particularly during treatment with repeated doses when plasma concentrations may increase, as M6G is not completely eliminated by HD **(Figure 7)**. Plasma concentrations of M3G and M6G were higher in EMs

compared with PMs. Also, within the EM genotype group, there was a 100-fold interindividual variation within both metabolites, which is in accordance with earlier studies of codeine and debrisoquine (41). In HD patients, variability of the CYP2D6 enzyme influences not only plasma concentrations and the effects of morphine but also those of M6G present during the whole interdialytic period. Our knowledge about the clinical use of codeine in patients with renal failure or HD patients is very limited, and so far, the recommendation is to not use codeine in such patients (80). Furthermore, there have been some case reports regarding serious side effects of codeine in patients with renal failure (81, 82). These are several factors that need to be taken into consideration when drugs metabolized by CYPs are prescribed to HD patients. Taking them into account may improve both the efficacy and safety of such drugs and reduce the risk of side effects. Further studies in larger dialysis populations are necessary to optimize the dosing of drugs metabolized by CYP enzymes in this patient population.

One limitation with the genotyping procedure is that it does not detect the CYP2D6*5 allele, where the entire gene is deleted. An individual with the *4/*5 genotype will with our method be detected as *4/*4, both genotypes are phenotypically PM i.e.no problem. With the used genotyping procedure, *1/*1 could not be distinguished from *1/*5, but they are both phenotypically EM. They have two and one genes, respectively, expressing the enzyme. Only about 5 % of Swedish subjects have this *5 allele (71) and only one out of 20 subjects thus carries this allele. Thus the limitation of our genotyping procedure has no significant effect on our results in study 1.

4.2.1 Results Study 2

After a single dose of alprazolam, the plasma concentrations of unconjugated alprazolam and metabolites decreased in all patients, and HD seemed to have no or only a minor effect on their plasma levels **(Fig.8, left)** or the metabolic ratio (MR) of unconjugated alprazolam to 4-hydroxyalprazolam (Fig. 9). The MR decreased from 10 to 34 h after drug administration (10/34 h: 1.5±0.6) which was similar to healthy individual (83). Figure 9 in this

section replaces the erratically published figure 3 in study 2.The published figure is shown here in Figure 9, but crossed over.

The average t½ for alprazolam in our 26 patients was 17 h (median 15.5 h, range 5–42 h), which is slightly longer compared to the 12 h that has been reported in normal individuals (84). There was an almost 40-fold interindividual variation in the concentration of unconjugated alprazolam at 38 h, i.e. after HD (0.97–36 nM). For unconjugated 4-hydroxyalprazolam and α-hydroxyalprazolam, the interindividual differences in the concentrations were about tenfold **(Fig. 8, left)**. The interindividual variation in extrapolated 0-h concentrations of unconjugated alprazolam was smaller than the variation in the 34-h concentration, indicating that there was no substantial variation in the drug absorption or first-pass metabolism among our patients. It was possible to measure the conjugated alprazolam in all patients. In healthy individuals, this metabolite is normally cleared very rapidly from plasma via the kidneys, but it remained unchanged in our study patients, who had little/no kidney function, until HD **(Fig. 8, right)**.

To the best of our knowledge, this is the first time that conjugated alprazolam has been measured in human plasma. The concentration of conjugated alprazolam was similar to that of conjugated hydroxy metabolites **(Fig. 8, right)**, and the concentrations of both conjugated alprazolam and its metabolites remained unchanged and even increased from 10 to 34 h after drug administration, i.e. the start of the HD session. During the HD session, their concentrations decreased dramatically **(Fig. 8, right)**, with the mean concentration of conjugated alprazolam decreasing by almost 80% ($P<0.0001$), and the mean concentrations of conjugated 4hydroxyalprazolam and α-hydroxyalprazolam decreasing by 75% ($P<0.0001$) and 68% ($P<0.0001$), respectively.

CYP3A4 activity, determined as the ratio of unconjugated AUC alprazolam to AUC 4-hydroxyalpazolam (MR) during the 10–34 h following drug administration, was significantly correlated ($r_s=0.49$, $P=0.011$, $n=26$) to the degree of inflammation (expressed as the median CRP of the last 5 weeks of the study) **(Fig. 10)**. This result shows that patients with a higher CRP

level had a higher ratio of unconjugated AUC alprazolam to AUC 4-hydroxyalpazolam, indicating lower activity of CYP3A4. Similarly, there was a significant correlation between the t½ of unconjugated alprazolam and median CRP level (r_S=0.39, P=0.047). We also found a significant correlation between the MR of alprazolam and the last measured CRP value (P=0.029). The ratio of unconjugated AUC alprazolam to AUC unconjugated α-hydroxyalprazolam showed, however, no significant correlation with CRP (r_S=0.03, P=0.89). The ratio of AUC unconjugated alprazolam to total 4-hydroxyalprazolam (conjugated + unconjugated) was significantly correlated to the CRP value (r_S=0.47, P=0.016), but the total α-hydroxyalprazolam was not (r_S=0.19, P=0.35). The ratio of 4β-hydroxycholesterol to cholesterol, which is an endogenous marker for CYP3A4 [18], was not significantly correlated to the median CRP value (P=0.87) in our group of patients. Alpha 1-acid glycoprotein (another marker for inflammation) and CYP3A4 activity were not significantly correlated to either alprazolam MR or 4βhydroxycholesterol/cholesterol. No major side effect was reported during the study.

Figure 8: Plasma concentration of both unconjugated (left) and conjugated (right) alprazolam and the metabolites α-hydroxy-alprazolam and 4-hydroxy-alprazolam. BHD and AHD = before and after hemodialysis.

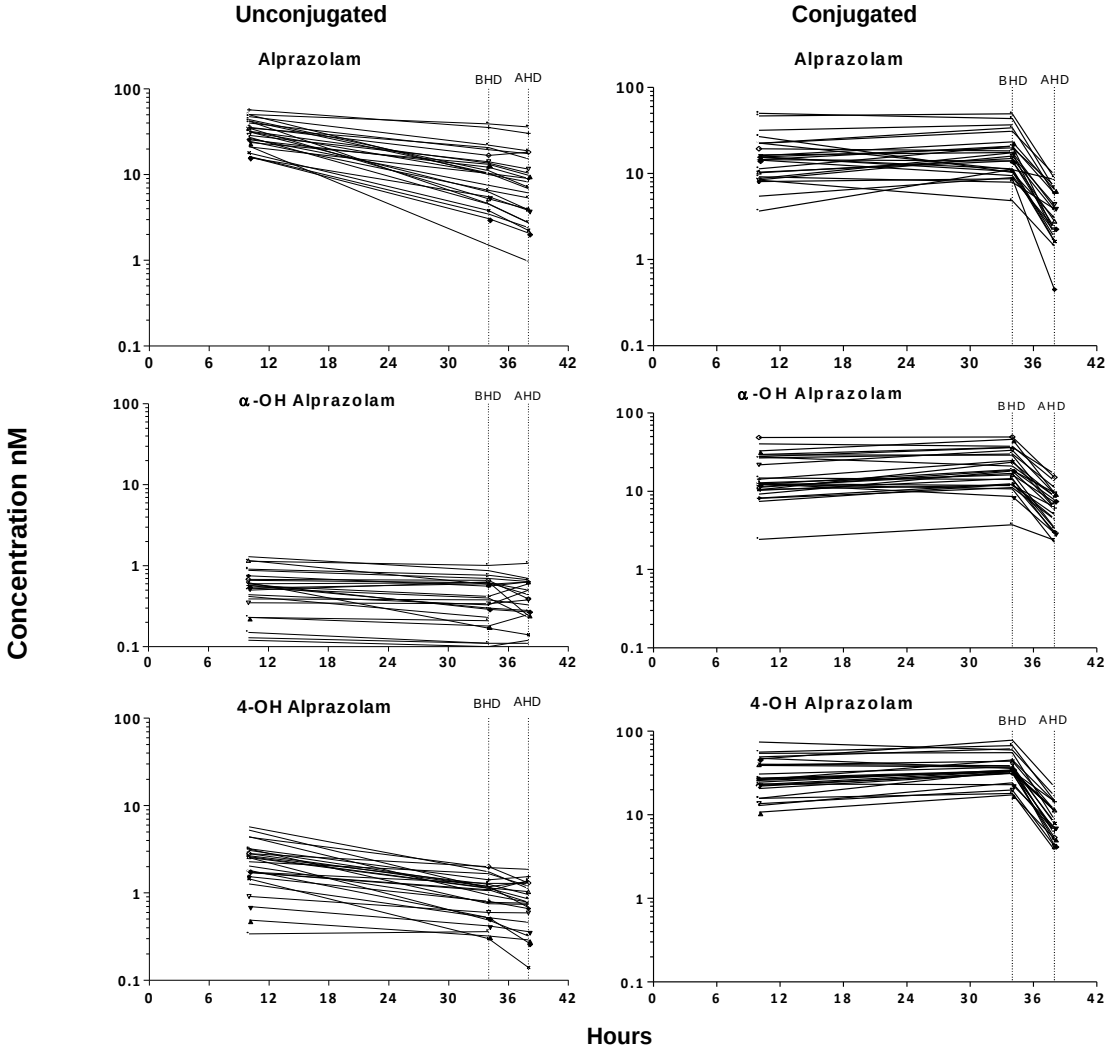
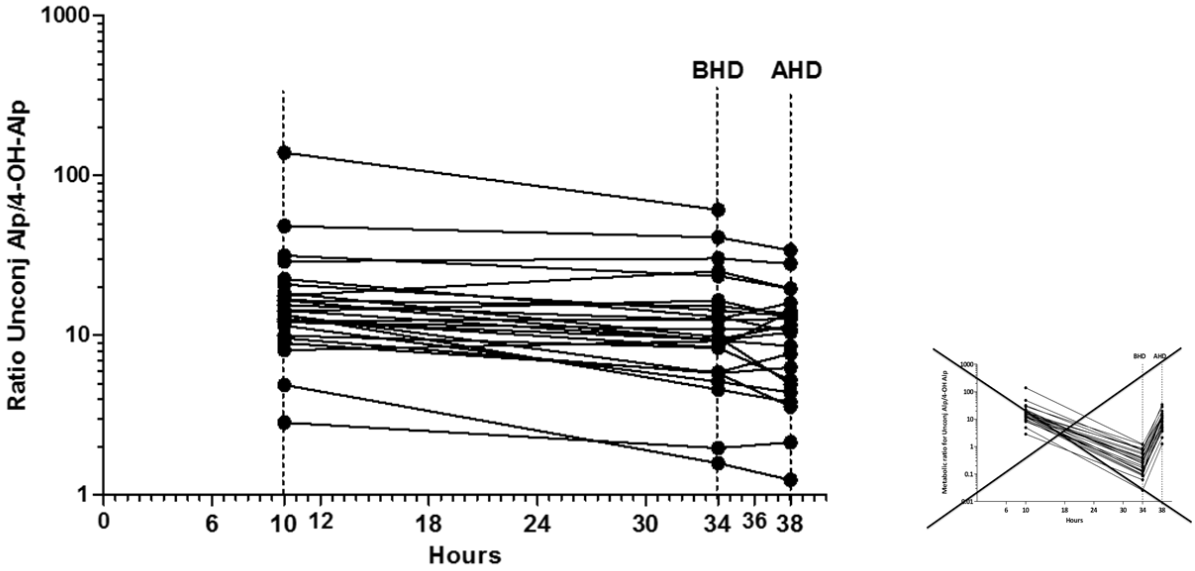
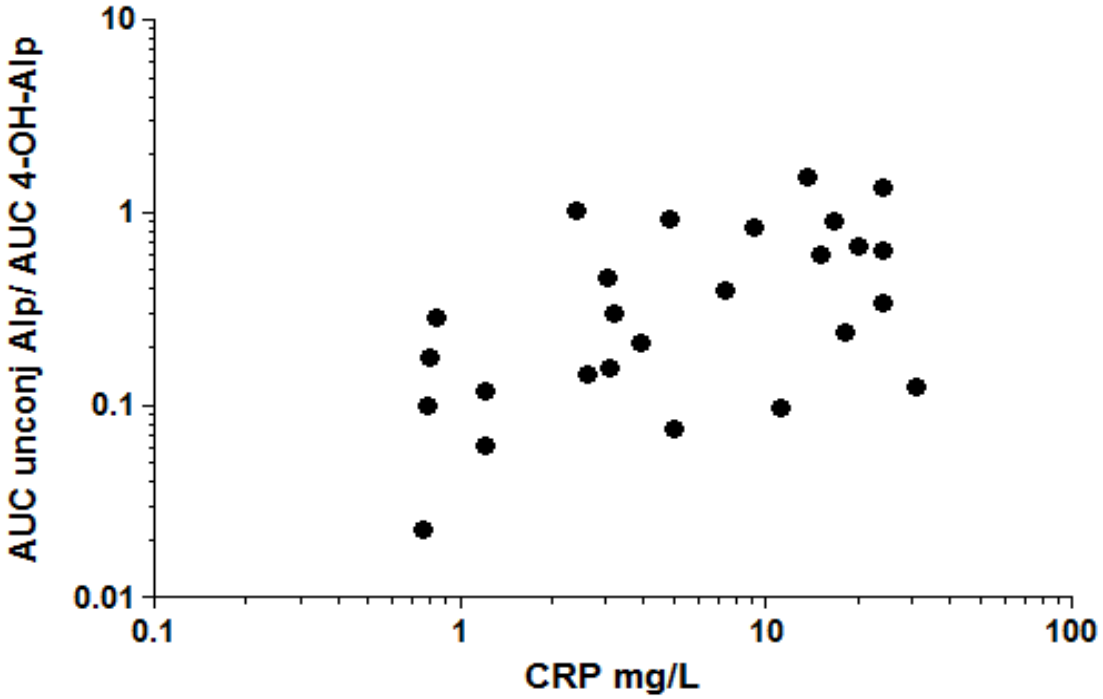


Figure 9: Changes in metabolic ratio of unconjugated alprazolam to unconjugated 4-hydroxyalprazolam from 10h to 34 h after drug administration and from 34 to 38 h after drug administration.



BHD: Before hemodialysis, AHD: After hemodialysis. Figure 3 in the published study 2 is erratic and therefor crossed over here.

Figure 10: Correlation between CYP3A4 activity expressed as the ratio for AUC of unconjugated alprazolam/AUC of unconjugated 4-hydroxy-alprazolam from 10h to 34h and median CRP (n=26, r_s=0.49, P=0.011).



4.2.2 Discussion study 2

As early as 1977, increased plasma protein binding of propranolol and chlorpromazine was shown to be mediated by disease-induced elevations of plasma alpha 1-acid glycoprotein, an acute-phase plasma protein (26). Several studies in recent years have shown that the expression of drug-metabolising enzymes (DME) and transporters is down-regulated by inflammation (85-89). Chronic inflammation in patients with cancer may cause similar changes in DMEs and transporters (86). A link between tumor-derived cytokines and the downregulation of CYP3A4 has been shown in tumor-bearing mice (86). HD patients represent a sensitive group of patients, and many have multiple complications, making it difficult to

participate in studies that include tests that are not included in their normal dialysis routine. Consequently, for ethical reasons we did not include more than three measurement moments in the design of our study. The patients only had to come to the hospital to leave the 10-h blood sample in addition to the routine visit the next day for HD as planned. The increasing concentration of conjugated alprazolam and its metabolites from 10 to 34 h following the administration of alprazolam **(Figure 8, right)** may be explained by the decreased kidney function of our patients, since conjugated compounds are very slowly eliminated by the kidneys of CKD patients. Newly conjugated compounds will be added to the conjugated alprazolam and metabolites already accumulating in the plasma and thereby still further increase their plasma concentration. Another possible explanation may be enterohepatic recirculation. The alprazolam conjugates reach the intestine via bile secretion and are subsequently hydrolysed to unconjugated compounds. They will then be reabsorbed from the intestine and reach the blood circulation. These unconjugated compounds can again be glucuronidated and be present in the plasma as conjugated alprazolam or metabolites. Such an extrahepatic recirculation would then prolong the elimination of the unconjugated compounds, resulting in their increased concentrations in plasma, which is indicated by the higher ratio for alprazolam and its metabolites. This in turn could result in the slightly higher t½ in these patients compared to healthy subjects (see above). Figure 9 shows 50% decrease in the MR of unconjugated alprazolam to unconjugated 4hydroxyalprazolam from 10 to 34 h after drug administration, similar to healthy individuals (83). Hemodialysis had no effect on unconjugated alprazolam, 4hydroxyalprazolam or their MR.

There was a significant correlation between CRP and ratio for alprazolam and its major metabolite 4-hydroxyalprazolam (83), indicating that inflammation could reduce the activity of CYP3A4 **(Figure 10)**. There was no significant correlation between CRP and the metabolic ratio of alprazolam and its other metabolite, α-hydroxyalprazolam. This could possibly be explained by the low concentrations of this metabolite and thereby more variability in the measurement of this α-hydroxy metabolite. There was no significant correlation between the metabolic ratio of alprazolam and the second surrogate marker of inflammation, alpha 1-acid

glycoprotein. One explanation may be that whereas CRP was used as a median value, calculated from the weekly measurements taken during the 5 weeks preceding drug intake, alpha 1acid glycoprotein was measured only once at baseline. Based on data collected before and at the time of drug intake, none of our patients had very high CRP levels, an indication of acute infectious processes. An important point is that we could demonstrate that even a relatively low degree of inflammation could reduce CYP3A4 activity. Further studies are needed to demonstrate if a higher degree of inflammation would reduce CYP3A4 activity even more. The AUC ratio of unconjugated alprazolam to unconjugated 4-hydroxyalprazolam might be a more suitable marker for CYP3A4 activity than the ratio of 4β-hydroxycholesterol to cholesterol or 4β-hydroxycholesterol itself in our group of patients. 4β-Hydroxycholesterol is a useful marker for determining CYP3A4 activity in healthy individuals (50) and for detecting the induction of this enzyme (90). This cholesterol metabolite is, however, very slowly eliminated after induction (44). If the degree of inflammation in our patients varies over time, this variation may not be reflected by changes in the metabolism of the slowly eliminated 4β-hydroxycholesterol. The rate of mortality is high among CKD patients (5), with cardiovascular disease (CVD) being the most common cause of mortality among this patient population. The high rate of CVD cannot be adequately explained by traditional risk factors, such as hypertension, dyslipidemia and smoking alone (91, 92); rather, non-traditional risk factors, such as inflammation and oxidative stress, seem to be more important in this group of patients (91, 93, 94). Several studies have shown an association between increased mortality and elevated CRP in both HD and PD patients (7). Inflammation has been shown to be a strong predictor of the number of atherosclerotic plaques in the carotid arteries of end stage renal disease patients, and a strong association has been found between the level of atherosclerosis and elevated CRP (95). In addition, the results reported here also shown that inflammation may also play an important role in drug metabolism.

The clinical consequence of reduced CYP3A4 activity is an increased plasma drug concentration at a certain dose. CKD and the uremic state itself could also alter the activity of some CYP enzymes (64), which would in turn have an additive effect on drug metabolism and

possibly increase the risks for drug accumulation and concentration dependent adverse drug reactions. This is also the risk associated with the decreased renal excretion of drugs. We cannot exclude the possibility that alprazolam glucuronides have some clinical effects, as previous studies have shown that some glucuronides (e.g. morphine glucuronides) have important clinical effects (96, 97). Another interesting finding of our study is that conjugated alprazolam can be quantitated in human plasma. To the best of our knowledge, this is the first report of conjugated alprazolam being measured in human plasma; in healthy individuals, this metabolite is normally excreted very rapidly from the plasma. This finding indicates that the renal clearance of this drug metabolite was reduced in our HD patients. At the present time, it is not known if conjugated alprazolam is active. In a previous study, however, we did demonstrate that the polar and active metabolite morphine-6-glucuronate could accumulate in dialysis patients (97).

4.3.1 Results study 3

Laboratory data in the 44 HD patients on the day of investigation, i.e., 12 hours after intake of a single dose of 100 mg quinine ("Day 2" in Table 6), and median values for hs-CRP, orosomucoid and PTX3, calculated from five samples taken during 4 weeks prior to the study plus on Day 2, are shown in **Table 8**. Significant correlations were observed between plasma albumin and both preceding time on dialysis, dialysis vintage (Rho=0.37; p=0.013) and age (Rho=-0.39; p=0.0085). As expected, inflammatory markers correlated with each other: median orosomucoid vs. median hsCRP (Rho=0.83; p<0.001), median orosomucoid vs. IL-6 (Rho=0.46; p=0.0016) and IL-6 vs. median hsCRP (Rho=0.45; p=0.0023).

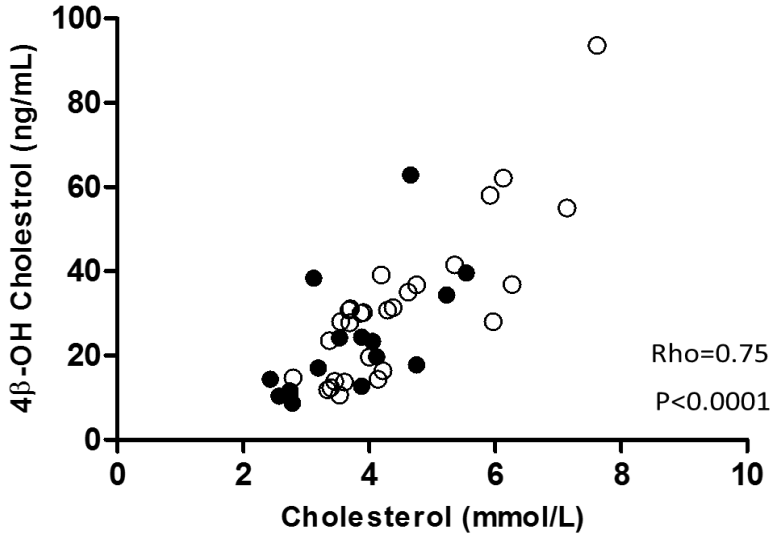
Table 8. Laboratory values in 44 HD patients on the final day of investigation (Week 5, Day 2; see Table 6) whereas values for inflammatory markers, hsCRP, orosomucoid and PTX3, are shown as median values over 4 weeks plus the final day of investigation.

Laboratory parameters	
hsCRP, mg/L ª	4.5 (1.5 - 14.0)
Interleukin-6, pg/ml	6.0 (3.0 - 10.6)
Orosomucoid, g/L ª	1.0 (0.8 - 1.3)
Pentraxin 3, ng/ml ª	1.3 (1.1 - 2.0)
B-type natriuretic protein, g/L	6330 (1285 – 21830)
Parathyroid hormone, ng/L	322 (151- 587)
Albumin, g/L	33 (32 - 36)
Iron, µg/L	10 (8 - 13)
4β-OH-Cholesterol, ng/ml	26 (14 - 26)
Cholesterol, mmol/L	3.9 (3.4 - 4.7)
Urea reduction rate, %	74 (70 - 78)
Hemoglobin, g/L	115 (106 - 121)

Data presented as median and interquartile range (IQR). ª Median values for high sensitive (hs) CRP, orosomucoid and pentraxin-3 are calculated from the five samples taken during 4 weeks prior to the study plus the last day of the study. The rest of the parameters are sampled at day 2 of week 5.

A significant correlation was also observed between 4-beta-OH-cholesterol and cholesterol (Rho=0.75; p<0.0001) **(Fig 11)**. As 15 of the 44 patients were treated with statins, we investigated this correlation in patients with or without statins **(Fig 11).** Significant correlations were observed both in patients on statins (Rho= 0.67; p=0.006) and not on statins (Rho=0.83; p<0.0001) **(Fig 11)**.

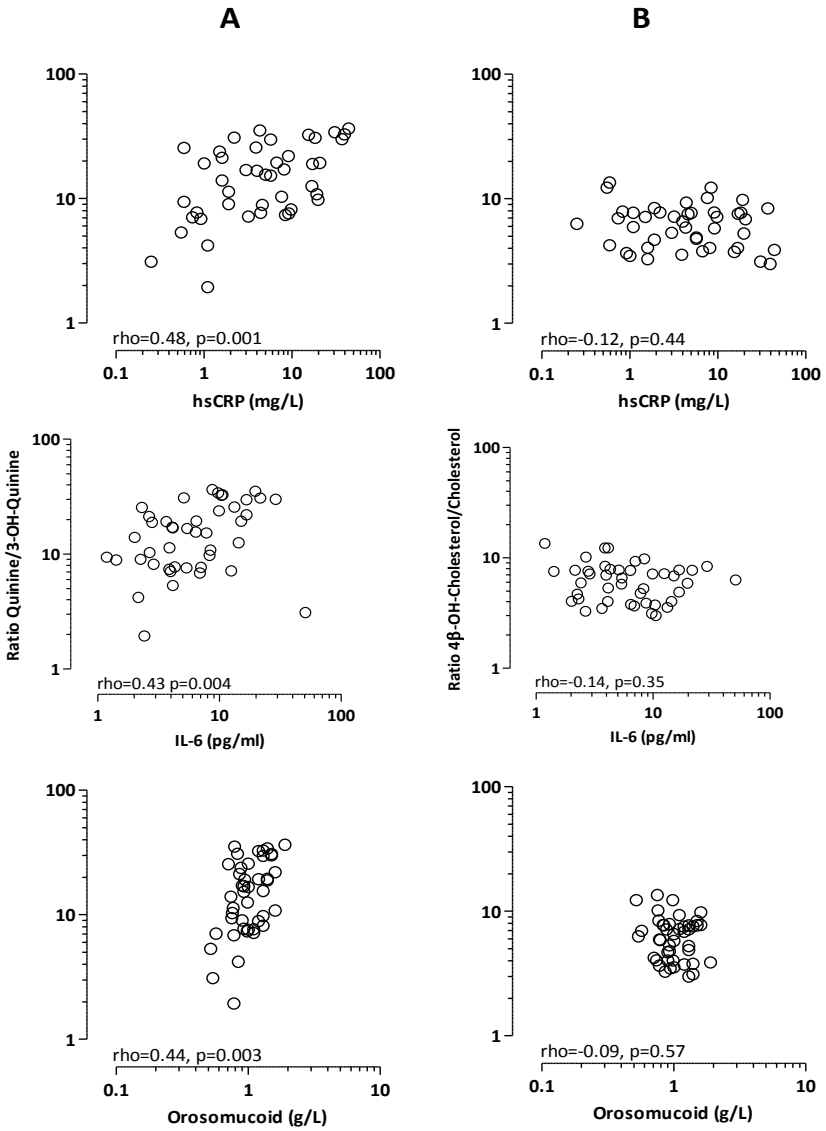
Figure 11: Correlation between 4-beta-OH-cholesterol and cholesterol. Closed circles represent 15 patients treated with statins and open circles represent 29 patients without statin treatment.



Significant correlations between CYP3A4 activity (expressed as ratio of quinine/3-OH-quinine) and median hsCRP (Rho=0.48; p=0.001), IL-6 at day 2 at the end of week 5 (Rho=0.42; p=0.004) and median orosomucoid (Rho=0.44, p=0.003) were observed (**Figure 12A**). Median values of hsCRP, PTX3 and orosomucoid were calculated from the measurements four weeks prior to the study plus the values taken at the last day of the study. Also the last value (samples taken at the same day as quinine) of hsCRP (Rho=0.59; p<0.001) and orosomucoid (Rho=0.45; p=0.002) correlated to the ratio of quinine/3-OH-quinine. We did not observe any correlation of the ratio of quinine/3-OH-quinine with pro-BNP (Rho=0.15; p=0.332,) and plasma albumin (Rho=-0.18; p=0.237), respectively. A significant correlation (Rho=-0.40; p=0.008) was also found between ratio of quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol; a marker of CYP3A4 activity. No correlations were observed between the ratio of 4β-OH-cholesterol/cholesterol and median hsCRP (Rho=-0.12; p=0.44), IL-6 (Rho=-0.14; p=0.35) and median orosomucoid (Rho=-0.09; p=0.57) (**Figure 12B**), respectively. We did not find any

correlation between median PTX3 and CYP3A4 activity expressed as either quinine/3-OH-quinine (Rho=-0.17; p=0.26) or 4β-OH-cholesterol/cholesterol (Rho=-0.21; p=0.16).

Figure 12: Correlation between CYP3A4 activity, expressed as the ratio of quinine/3-OH-quinine (A) and 4β-OH-cholesterol/cholesterol (B), respectively, and median concentrations of hsCRP and orosomucoid as well as with a single measure of IL-6 (on Day 2 at week 5).



The association between CYP3A4 activity expressed as quinine/3-OH-quinine and hsCRP showed a trend to be significant (β=0.44; p=0.05) in a multivariate analysis after full adjustment for age, gender, diabetes mellitus, dialysis vintage, PTH, orosomucoid and medication with angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), beta-blockers or statins **(Table 9)**.

Table 9: Multiple regression models for determinants of the ratio quinine/3-0H-quinine in 44 prevalent hemodialysis patients. Values are expressed as beta (β) and significance.

	Unadjusted (β, *P*) (r^2=0.23)	**Model 1 (β, *P*) (r^2=0.27)**	**Model 2 (β, *P*) (r^2=0.27)**	**Model 3 (β, *P*) (r^2=0.21)**
hsCRP (mg/l)	0.49 (**0.001**)	0.52 (**0.001**)	0.54 (**0.001**)	0.44 (**0.05**)
Age (years)		0.02 (0.90)	-0.002 (0.98)	-0.03 (0.85)
Gender (female)		0.28 (**0.04**)	0.27 (0.07)	0.30 (0.07)
Diabetes mellitus		-0.13 (0.32)	-0.18 (0.22)	-0.14 (0.44)
Vintage (months)		-0.11 (0.42)	-0.10 (0.50)	-0.08 (0.62)
Betablockers			0.14 (0.38)	0.12 (0.46)
ACEi/ARBs			-0.21 (0.19)	-0.21 (0.21)
Statins			0.18 (0.31)	0.18 (0.31)
PTH (ng/L)				-0.01 (0.93)
Orosomucoid (g/L)				0.14 (0.56)

Abbreviations: hsCRP: high sensitivity C reactive protein, ACEi: angiotensin converting enzyme inhibitors, ARBs; angiotensin receptor blockers, PTH: parathyroid hormone,

4.3.2 Discussion study 3

In the present study, a single dose of 100 mg quinine was given to 44 ESRD patients undergoing maintenance HD and plasma quinine and its metabolite 3-OH-quinine were measured after 12 hours immediately prior to the next HD session. Patients with signs of inflammation - according to weekly measurements of several biomarkers of inflammation over 4 weeks prior to the investigation – had a higher quinine/3-OH-quinine ratio, indicating decreased CYP3A4 activity, suggesting that the activity of CYP3A4 is reduced by inflammation in HD patients.

As kidney failure can alter the pharmacokinetics of many drugs at different levels (56, 98), drug dosing has always been a challenge in this vulnerable patient population (99). The absorption of orally administered drugs in the gastrointestinal system may be affected by reduced gut motility, increased pH, increased paracellular transport across the intestinal epithelium (100) and reduced activity of drug metabolizing enzymes and transporters (64, 101). This may result in increased rate of absorption and consequently increased bioavailability of drugs. The absorbed drugs might be bound to albumin or other plasma proteins during the transport to the target organ e.g. liver. However, as the concentrations of plasma proteins, including albumin, in general are decreased in ESRD patients, protein binding of drugs is reduced, and the free circulating concentration of the drug may therefore increase (102). Furthermore, the uremic milieu *per se* could reduce the non-renal elimination of drugs by affecting the function of drug metabolizing enzymes and transporters leading to even larger risk of drug accumulation and drug intoxication (64).

As dialysis patients are often subjected to polypharmacy this increases the risk of drug-drug interactions (103, 104). Furthermore, the concentration of circulating drugs is not only affected by the changes in drug metabolism and pharmacokinetics, but also by the dialysis treatment *per se*. The dialyzability of a drug depends on several factors, such as molecular weight, protein binding, volume of distribution, blood and dialysis flow rates during the dialysis treatment, and type of the dialysis membrane (98, 105). Another important observation is that

HD is reported to increases the metabolic activity of CYP3A4 suggesting that dialyzable uremic toxins may inhibit the activity of this enzyme (65, 106). Thus, nephrologists need to consider a myriad of factors when a drug is prescribed to a dialysis patient. Further, as inflammation may alter the activity of drug metabolizing enzyme and transporters (107), this condition, which is common in dialysis patients may add to the difficulties to prescribe drugs (28).

So far most studies on the impact of inflammation on drug metabolism have been conducted in animal models (107, 108). In the current study, we studied pharmacokinetics of quinine, a substrate for CYP3A4, in a group of HD patients. Quinine is commonly used in dialysis wards against leg cramps in HD patients during the dialysis treatment and the risk of side effects is small for current doses prescribed to HD patients (100-250mg). Our results show that CYP3A4 activity associate to biomarkers of inflammation. Since the already complicated drug metabolism in dialysis patients may be affected also by systemic inflammation, this adds novel challenges to correct drug dosing in this inflamed patient population (15). Further, as Shah and Smith (109) reported that phenoconversion of drug metabolizing enzymes may be an important modifier of drug metabolism, the scenario may be even more complicated. Since inflammation may induce phenoconversion (110) this implies that genetically extensive metabolizers could be converted to a phenotypic poor metabolizer. Clearly, we need to individualize drug dosing to reduce the complications related to drug side effects and interactions. For this purpose, we need to identify which factors in addition to traditionally known factors that affect pharmacokinetics and pharmacodynamics of drugs.

The results of the present study should be considered in light of the following strengths and caveats. The careful repeated monitoring of inflammation biomarkers during four weeks preceding the pharmacokinetic study of quinine strengthens the ascertainment of the inflammatory burden of the investigated patients. Some caveats deserve mentioning. As the sample size is rather small, the results need to be confirmed in larger cohorts. In a prior study, using alprazolam as a test drug, we demonstrated that inflammation associated to reduced activity of CYP3A4 in another cohort of HD patients (111). Since orosomucoid can be considered as a confounder due to its ability to bind drugs (26), thereby raising the plasma

concentration of quinine, this could lead to misinterpretation of reduced CYP3A4 activity. However, the correlation between quinine/3-OH-quinine and hsCRP showed a trend to being significant (p=0.05) after multivariate regression analysis **(Table 9)** implying that inflammation *per se* affect the activity of CYP3A4. In the present study, no correlation between CYP3A4 activity and PTX3 was observed. However, it has been reported that PTX3 may primarily reflect endothelial dysfunction rather than systemic inflammation (112). Another limitation is that the impact of accumulation of uremic toxins and its putative effects on drug functions and metabolism was not assessed.

Although we report a significant correlation between CYP3A4 activity expressed as quinine/3-OH-quinine and the ratio of 4β-OH-cholesterol/cholesterol; the latter being another marker for CYP3A4 activity (44), no correlation between the inflammatory biomarkers and the ratio of 4β-OH-cholesterol/cholesterol was observed. This finding is in accordance to our prior study using alprazolam as test drug (111). Although 4β-hydroxycholesterol is considered to be a useful marker for CYP3A4 activity in healthy individuals, this marker may not be suitable for detecting CYP3A4 activity in HD patients. One reason could be that this cholesterol metabolite is very slowly eliminated after induction and therefore the relatively faster variation of the degree of inflammation may not be reflected by changes in metabolism of the slowly eliminated 4β-OH-cholesterol (111). CYP3A accounts for 80% of total P450 content in intestines (113), and although the CYP3A content in intestine is only about 1% of the amount in the liver, its predominance in human intestine can lead to several fold more efficacy of the enzyme in intestine compared to liver (114, 115). Indeed, the intestine is suggested to be of equal or even greater importance than liver for metabolism of drugs (116). Furthermore, since the inflamed uremic milieu is associated with changes in gut microbiota (61), the discrepant findings with regard to the relation of quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol with inflammation could imply a role of uremic dysbiosis on the pharmacokinetic profile (117). In a previous study in 440 healthy subjects representing three major populations in Africa, Asia and Europe, the coefficient of correlation between 4β-OH-cholesterol and cholesterol was low (R=0.30), but significant (p<0.0001); i.e. only 9% of the

variation in 4β-OH-cholesterol concentration was due to the variation in cholesterol concentration (50). Thus, a major determinant of the level of 4β-OH-cholesterol might be the CYP3A4 activity and not the concentration of the substrate i.e., cholesterol. In the present study, we found a much stronger correlation (rho=0.75); fairly independent of statin treatment **(Fig 11)**. In our previous study using alprazolam as a marker of CYP3A4 activity, we also found a fairly high coefficient of correlation between 4β-OH-cholesterol and cholesterol (Rho=0.58; p=0.0018) (calculated from data of ref 12). In these two studies on HD patients the variation in 4β-OH-cholesterol concentration is determined to a pronounced extent by cholesterol (56 % in the present study and 34% in (111)), which is higher than the 9% reported in healthy subjects (50). We propose that 4β-OH-cholesterol/cholesterol is a better marker of CYP3A4 activity in healthy subjects while it is an inadequate marker in HD patients which could explain the absence of a relationship between CYP3A4 activity measured by 4β-OH-cholesterol and markers of inflammation in two independent groups of HD patients investigated by our group. Further Björkhem-Bergman et al (118) have shown that whereas statin treatment had no effect on the hepatic CYP3A mRNA content, it significantly reduced 4β-OH-cholesterol, while there was no significant effect on the 4β-OH-cholesterol/cholesterol ratio (119). An earlier *in vitro* study showed that the CYP3A4 enzyme is saturated at a cholesterol concentration of 100 µM [19]. Both 4β-OH-cholesterol and cholesterol are transported in lipoproteins in the circulation (74). These data indicate that during statin treatment it is mainly the cholesterol-dependent lipoprotein binding capacity in the circulation that will determine the 4β-OH-cholesterol concentration in plasma, rather than a direct effect on the hepatic CYP3A4 enzyme. A disturbed cholesterol-dependent lipoprotein binding capacity in HD patients may also be operative (120). Taken together, our results suggest that the ratio 4β-OH-cholesterol/cholesterol rather than 4β-OH-cholesterol alone, is the preferred measure of CYP3A4 activity.

4.4.1 Results study 4

At inclusion the concentrations of 25-OH-vitamin D_3 at study start were <50 nmol/L in all 8 patients which according to KDOQI guidelines is considered as vitamin D insufficiency (69). The results of measured values for the 8 remaining patients who completed the study are given in **Table 10**. Whereas the levels of 25-OH-vitamin D_3 increased after supplementation (40.1±10.0 nmol/L to 71.0±10.4 nmol/L, P=0.0003) **(Figure 13A)** no significant changes were observed in 1,25-OH-vitamin D_3 levels (14.5±6.5 ng/L to 19.5±4.3 ng/L, P=0.12) **(Figure 13B)**.

Figure 13: Changes in the concentrations of 25-OH-vitamin D_3 and 1,25-dihydroxyvitamin D_3 before and after 25-OH-vitamin D_3 supplementation in all 8 patients who completed the study.

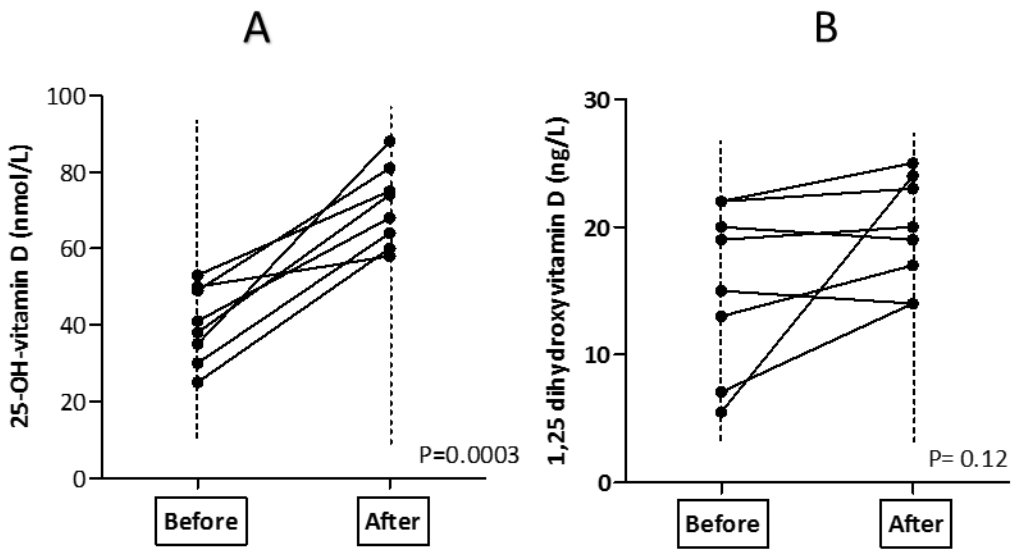


Table 10. Laboratory values before and after 25-OH-vitamin D_3 supplementation in 8 HD patients who completed the study.

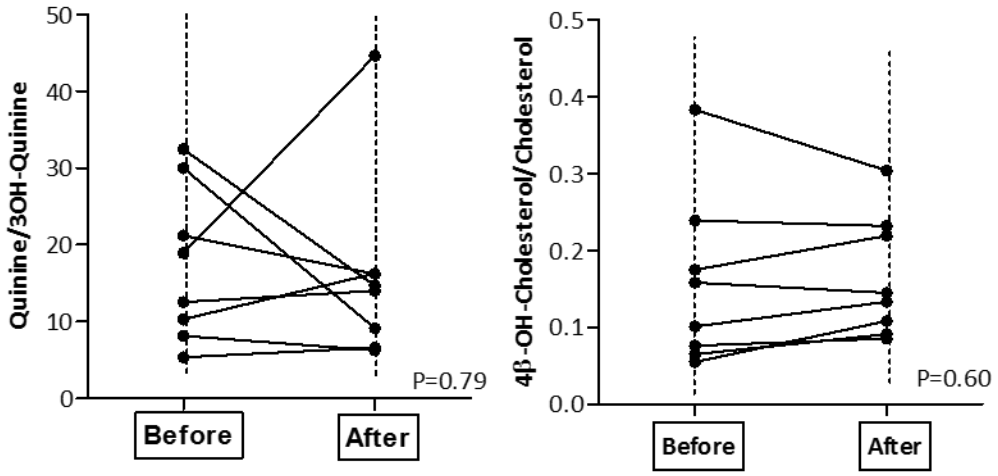
Laboratory parameters	Before 25-OH-vitamin D_3 supplementation	After 25-OH-vitamin D_3 supplementation	P-value
hsCRP, mg/L	7.5 (1.0-19.5)	5.2 (0.9-42.0)	NS
Orosomucoid, g/L	1.0 (0.6-1.3)	1.0 (0.5-1.9)	NS
25-hydroxyvitamin D_3 nmol/L	40 (25-53)	71 (58-88)	0.0003
1,25 hydroxyvitamin D_3 ng/L	17 (6-22)	20 (14-25)	NS
NT-Pro-BNP, ng/L	7779 (1070-20800)	16500 (1280-35000)	0.046
Parathyroid hormone, ng/L	25.3 (1.7-105.0)	38.0 (0.4-55.0)	NS
Albumin, g/L	34 (30-39)	34 (22-42)	NS
Iron, µg/L	11 (6-13)	12 (7-22)	NS
4β-OH-Cholesterol, ng/ml	21.3 (9.3-53.6)	20.0 (9.6-48.5)	NS
Cholesterol, mmol/L	3.8 (3.0-5.6)	3.6 (2.8-5.2)	NS
Urea reduction rate, %	74 (56-80)	74 (63-78)	NS
Hemoglobin, g/L	117 (98-122)	120 (104-121)	NS

Data are expressed as median and range, NS: non-significant

At study start we did not find any association between the 25-OH-vitamin D_3 levels and CYP3A4 activity expressed as ratios of quinine/3-OH-quinine (rho=-0.31, P=0.46) or 4β-OH-cholesterol/cholesterol (rho=-0.05, P=0.93). The mean ratios of quinine/3-OH-quinine (17.4±10.1to 16.0±12.3, P=0.79) 4β-OH-cholesterol/cholesterol (0.16±0.11 to 0.17±0.08, P=0.60) did not change significantly during 25-OH-vitamin D_3 supplementation **(Figure 14)**. The correlation between CYP3A4 activity expressed as quinine/3-OH-quinine (rho=0.69, P=0.07) or 4β-OH-cholesterol/cholesterol (rho=-0.31 P=0.46) and vitamin D remained non-significant after 25-OH-vitamin D_3 supplementation. We found no association between CYP3A4 activity

expressed as either quinine/3-OH-quinine or 4β-OH-cholesterol/cholesterol and hsCRP, orosomucoid, NT-Pro-BNP, hemoglobin and albumin. NT-Pro-BNP increased significantly (P=0.046) during cholecalciferol supplementation.

Figure 14: CYP3A4 activity expressed as quinine/3-OH-quinine and 4β-OH-cholesterol/cholesterol before and after 25-OH-vitamin D_3 supplementation.



4.4.2 Discussion study 4

In this study we investigated the correlation between CYP3A4 activity and 25-OH-vitamin D_3 levels after 25-OH-vitamin D_3 supplementation. Our result shows no association between 25-OH-vitamin D_3 supplementation and CYP3A4 activity, which is in accordance with prior studies (121).

Additional studies are needed to investigate this possible association further. We did not find any correlation between 4β-OH-cholesterol/cholesterol and CYP3A4 activity determined by quinine. As previously discussed (not published), quinine/3-OH-quinine seemed to be a better marker than 4β-OH-cholesterol/cholesterol for CYP3A4 activity in this group of

patients. Unexpectedly, we observed a significant increase of NT-Pro-BNP despite the short duration of vitamin D supplementation. NT-pro-BNP is an end-product from BNP (brain natriuretic peptide) which is released from heart ventricle. The reason for this increase is unclear. However, increased plasma level of BNP with vitamin D supplementation has been observed in the PRIMO study (122) a finding that disappeared after adjustment for eGFR (estimated glomerular filtration rate). Therefore, it is likely that loss of residual renal function during the study may account for the observed increase in NT-pro-BNP in our study.

The results of the present study should be interpreted with the following caveats in mind. Since the number of patients was limited we cannot exclude a type-2 statistical error. Moreover, the observation and treatment period was rather short. Thus, larger and long-term studies are needed to resolve if 25-OH-vitamin D_3 supplementation affect CYP-enzymes and drug pharmacokinetics. Another limitation is that we (for ethical reasons) could not study dialysis patients without ongoing treatment with active vitamin D (1α-hydroxyvitamin D). According to local protocols active vitamin D treatment is given to the majority of dialysis patients. It should also be noted that the serum levels of 25-OH-vitamin D_3 were only modestly decreased in our study (mean level 40 nmol/L), and it is possible that a more pronounced vitamin D deficiency would have had more effect on CYP3A4 activity. The adequate 25-OH-vitamin D_3 levels have been controversial and the limit for vitamin D deficiency is sometimes as low as 25 nmol/L, a level that no patient in our study met.

5. Conclusions

5.1 Study 1

Codeine disposition is affected by both CYP2D6 polymorphism and the HD procedure in patients with ESRD. PMs do not express the CYP2D6 enzyme and are therefore unable to metabolize codeine to the active metabolites morphine and M6G. Accordingly, we showed that concentrations of M3G and M6G are much higher in EMs compared with PMs. An important observation is that concentrations of glucuronidated metabolites in HD patients are unchanged or even increased until the patients undergo HD, which dramatically reduces the concentration of these metabolites.

5.2 Study 2

The renal elimination (excretion) of some drugs and metabolites is decreased in HD patients, but the rate of drug metabolism might also be decreased in these patients due to inflammation. Our study shows that even a low degree of inflammation (expressed as increased median CRP) can significantly reduce CYP3A4 activity. In all of the patients enrolled in our study, the conjugated metabolites remained at high levels until HD, which reduced the concentrations of conjugated alprazolam and metabolites. However, the level of unconjugated compounds or the metabolic ratio of unconjugated alprazolam to 4-hydroxyalprazolam was not effected by HD. Larger studies are needed to investigate if more severe inflammation has more profound effects on drug metabolism in this patient group as well as other persistently inflamed patients.

5.3 Study 3

In summary, a higher degree of inflammation associates with decreased activity of CYP3A4 in HD patients. Further studies are needed to find out if this consequence of inflammation will have a clinically significant impact on risk of drug interactions and side effects in dialysis patients.

5.4 Study 4

Our result shows no association between 25-OH-vitamin D_3 supplementation and CYP3A4 activity, which is in accordance with prior studies.

7. Strength and limitations

These studies are a small step for investigating the factors affecting the pharmacokinetics of drugs in HD patients. ESRD is a complicated disease affecting several aspect of pharmacokinetics. Such studies must be designed according to ethical considerations and patient's health and integrity. We were therefore limited the dose and number of test drugs and blood tests in the study. We have chosen drugs (codeine, alprazolam, quinine and 25-OH-vitamin D_3) often used in HD patients. We have also limited our study to small numbers of participants in the study leading to weaker power. Drug metabolism could be affected not only by inflammation but also by the uremic state per se. Furthermore HD patients are taking several drugs, which increase the risk of drug- drug interaction and possibly the concentration of test drug and its metabolites. Another important limitation was the lack of a control group, which was difficult to achieve due to limited number of HD patients who were willing to participate in the clinical studies. Some other factors could be the time of dialysis treatment, type of the dialysis filter/membrane and the effectivity of the dialysis treatment. We have tried to avoid these effects by administration the test drug or taking the blood test after and before dialysis treatment respectively.

8. Future perspectives

In the future we can design similar studies in larger groups of patients with different levels of inflammation. We can study the drug metabolism using some of the most common drugs (substrate of CYP3A4) that HD patients are using on daily basis. The patients could be their own control group by repeating the same drug test after the improving from the infection/ inflammation.

The same type of studies could be done before and after the start of dialysis treatment. Another important group that could be subject for these kind of studies is patients undergoing PD. Their treatment is on a daily basis and many of them have some residual function which could affect the pharmacokinetics of the drug. Study of drug metabolism in this group could also give light on this subject and be very beneficial for patients with ESRD.

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